

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:  
Joseph M. Penninger  
Michael A. Crackower

Serial No.: 10/518,599

Filed: May 31, 2005

For: ACE2 ACTIVATION FOR TREATMENT OF  
HEART, LUNG AND KIDNEY DISEASE  
AND HYPERTENSION

Group Art Unit: 1632

Examiner: Anoop Kumar Singh


Atty. Dkt. No.: SONN:064US

CERTIFICATE OF ELECTRONIC TRANSMISSION  
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

March 20, 2007

Date

  
Travis M. Wohlers

**DECLARATION OF DR. NIKOLAUS NEU UNDER 37 C.F.R. § 1.132**

I, Nikolaus Neu, hereby declare as follows:

1. I am an Austrian citizen residing in Innsbruck, Austria. I am the head of the pediatric intensive care unit at University Hospital Innsbruck. I have extensive research experience in the fields of cardiology and immunology. I have published scientific papers on topics such as acute pulmonary arterial hypertension in acute lung injury, peptide-induced inflammatory heart disease, and heart disease linked through antigenic mimicry. A copy of my *curriculum vitae* is attached as Exhibit 1.

2. I have reviewed the specification of the above-reference application, the amended set of claims, and the Office Action dated October 20, 2006 ("the Action"). I understand that the Action rejected claims 67-69, 73, and 98-103 for lack of enablement and for failure to comply with the written description requirement. I do not find this to be the case based on my review of the specification.

3. The present specification discloses that ACE2 is a critical negative regulator of the renin-angiotensin system (RAS) (paragraph bridging pages 2-3). ACE2 cleaves angiotensin I (Ang I) to generate Ang 1-9, and it cleaves angiotensin II (Ang II) to generate Ang 1-7 (Specification, p. 29, ln. 10-16). The effects of Ang II are summarized in the attached review articles by Danilczyk *et al.* (2004 and 2006) (attached as Exhibits 2 and 3, respectively). Therein it is reviewed that Ang II is a vasoconstrictor, promotes cardiomyocyte hypertrophy, fibroblast proliferation, cardiac and cardiomyocyte contractility and regulates glomerular hemodynamics - whereas Ang 1-7 is a vasodilator, inhibits cell growth, regulates sodium and water flux and reduces glomerular filtration (e.g. Danilczyk *et al.*, 2006, table p. 465). As mentioned above, Ang II is converted to Ang 1-7 by ACE2 thereby reducing the effects of Ang II and increasing the effects of Ang 1-7. Loss of ACE2 results in an increase in Ang II, which was shown in the mouse knock-out model in the present specification (Specification, p. 38, ln. 13-26).

4. The present specification discloses that various cardiac, lung, and kidney diseases are associated with an ACE2 decreased state (*see e.g.*, p. 2, ln. 28 to p. 3, ln. 6). This disclosure is supported by rat and mouse model studies. For example, decreased ACE2 mRNA and protein levels were observed in the kidneys of a hypertensive rat model (Specification, p. 32, ln. 21 - p. 33, ln. 22). The specification also describes an ACE2 knockout mouse, which is used to model

the ACE2 decreased state. In studies on the ACE2 knockout mouse, it was observed that loss of ACE2 leads to detrimental effects in the kidneys (p. 36, ln. 8-11), heart defects (p. 36, ln. 14 – p. 38, ln. 12), and increases the susceptibility of the lungs to injury (p. 40, ln. 12-20). The specification teaches that an ACE2 decreased state, such as cardiac, lung, and kidney disease, may be treated by administering to an animal in need thereof an effective amount of an agent that can increase the expression of ACE2 (p. 9, ln. 10-15). The specification further teaches that this agent may be an Ace2 protein or fragments thereof (p. 9, ln. 16-25).

5. The specification also discloses the evolutionary conservation of ACE2 structure and activity among mammals, as well as other organisms. FIG. 1A shows an alignment of the amino acid sequences of ACE2 from human, rat, and mouse, which illustrates the identities and similarities between these sequences. Previous results in *Drosophila* showed that a P-element mutation associated with the ACE homologue, ACER, results in a severe and lethal defect of heart morphogenesis, which is further evidence that ACE/ACE2 functions in the heart have been conserved through evolution (Specification, p. 30, ln. 28 to p. 31, ln. 2). I have also reviewed a publication entitled “Structure, Evolutionary Conservation, and Function of Angiotensin- and Endothelin-Converting Enzymes” (Macours *et al.*, *International Review of Cytology*, 239:47-97 (2004); IDS reference C63), and the results of a BLAST search (IDS reference C60) of the ACE2 substrate, Ang II, which shows that Ang II is present in numerous mammals including *Pan troglodytes*, *Mus musculus*, *Homo sapiens*, *Callithrix jacchus*, *Gorilla gorilla*, *Canis familiaris*, *Macaca mulatta*, *Rattus norvegicus*, *Ovis ammon*, and *Pongo pygmaeus*. The Macours *et al.* publication and the BLAST search results provide further evidence of the evolutionary conservation of ACE2 and its substrate Ang II. In view of this evidence, I find that ACE2

structure and function is conserved among mammals, and I would expect that the currently claimed method could be practiced in any mammal.

6. The use of protein therapy in the treatment of diseases is well-known in the medical field. The publication *Scientific Considerations Related to Developing Follow-On Protein Products*, 2004, which is cited in the present Office Action, illustrates this point. For example, this publication mentions the drugs Epogen®, which is a protein therapy based on human erythropoietin; and Neupogen®, which is a protein therapy based on granulocyte colony-stimulating factor (*see*, p. 1, 2<sup>nd</sup> para.). As a further example, the publication notes that six companies manufacture FDA-approved versions of human growth hormone (paragraph bridging pages 5-6). Since ACE2 is an endogenous protein in mammals and the present specification discloses the physiological role of ACE2, it would require only routine clinical studies to administer a therapeutically effective amount of an ACE2 polypeptide to a mammal having hypertension, congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, arteriosclerosis, renal failure, and/or lung disease, in order to treat the mammal as recited in the current claims.

7. I have also reviewed the reference by Imai *et al.* (*Nature*, 436:112-116 (2005); IDS reference C61), which further demonstrates that scientists can practice the currently claimed method based on the information provided in the present specification. Imai *et al.* showed that injecting ACE2 knockout mice or acid-treated wild-type mice with a recombinant human ACE2 protein protected the mice from severe acute lung injury (p. 112, col. 2; Figures 2(d)-(f)). Like the examples in the present specification, Imai *et al.* used an ACE2 knockout mouse model. Imai *et al.* also used a lung elastance assay as described in the specification (*see* p. 40, ln. 12-20), and

a route of administration (intraperitoneal injection) as disclosed in the specification (*see* p. 21, ln. 27-30). Thus, Imai *et al.* demonstrated a method of treating an ACE2 decreased state by administering to a mammal a therapeutically effective amount of ACE2 polypeptide. The results of Imai *et al.* also provide additional evidence of the conserved function of ACE2 and the renin-angiotensin system because a human ACE2 protein was able to complement ACE2 function in mice.

8. Studies by my research group at University Hospital Innsbruck provide additional evidence that the currently claimed method can be practiced without undue experimentation. Attached to this declaration as Exhibit 4 is a research report (“Research Report”) of work conducted by Alexander Löckinger and Benedikt Tremel of my research group. This work was pharmacologically evaluated by Manfred Schuster and Hans Loibner of the firm Apeiron for which this work was conducted. This report describes a study of recombinant human soluble ACE2 (rhACE2) in a piglet acute respiratory distress syndrome (ARDS) model.

9. The piglet ARDS model is a generally accepted animal model for the study of acute respiratory distress syndrome. ARDS was induced by continuous infusion of 50 µg/kg lipopolysaccharide (LPS) for the duration of the experiment and further 1 - 3 LPS bolus injections of 50 µg/kg each (Research Report, p. 1, para. 2). The average LPS quantity administered was 319 µg/kg and nearly equally distributed over both groups (Research Report, p. 1, para. 2). An ACE2 polypeptide, rhACE2, was administered as a central venous bolus injection at a dose of 100 µg/kg following the last LPS bolus injection and 120 minutes from the start of the continuous LPS infusion (Research Report, p. 1, para. 2). Intravenous injection is a route of administration disclosed in the present specification (*see* p. 21, ln. 27-30). The rhACE2 bolus

injections were well tolerated and did not show any apparent side effects (Research Report, p. 1, para. 3). Several hemodynamic parameters as well as pharmacokinetics were investigated in the piglet ARDS model.

10. Initial studies showed that rhACE2 had a half-life time in the piglet ARDS model of 77 minutes (Research Report, p. 1, para. 4; Figure 1).

11. Following treatment with rhACE2, pulmonary arterial pressure (PAP) stabilized or even decreased slightly in the rhACE2 treated group, while the control group showed a nearly 15% increase in PAP (Research Report, p. 2, para. 1; Figure 2). Systolic arterial pressure (SAP) was also measured. The control group showed an increase in SAP up to 12%, whereas after rhACE2 injection a stabilization and 5% decrease in SAP was observed (Research Report, p. 2, para. 2; Figure 3). The difference between the control and rhACE2 treatment groups was significant (Research Report, p. 2, para. 2).

12. Oxygen concentration was measured in arterial and venous blood samples taken from the piglets every 30 minutes (Research Report, p. 3, para. 1). Values are displayed in Figure 4 of the Research Report. Oxygen concentration decreased in arterial and venous blood in both groups (Research Report, p. 3, para. 1; Figure 4). A potential stabilization of arterial as well as venous oxygen concentration in the group receiving rhACE2, which might be observed first in the venous, later in the arterial blood, did not reach statistical significance in this study and will have to be confirmed in further experiments.


13. In view of the rat and mouse animal model studies in the specification showing the role of ACE2 in cardiac, lung, and kidney diseases; the disclosed similarities of the rat, mouse, and

human ACE2 structure and function; and the teaching in the specification that an ACE2 decreased state, such as cardiac, lung, and kidney disease, may be treated by administering to an animal in need thereof an effective amount of an agent that can increase the expression of ACE2; I believe that at the time the application was filed the inventors of the present application were in possession of a method of treating an ACE2 decreased state comprising administering to a mammal having hypertension, congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, arteriosclerosis, renal failure, and/or lung disease, a therapeutically effective amount of an ACE2 polypeptide.

14. Furthermore, in view of the rat and mouse animal model studies in the specification showing the role of ACE2 in cardiac, lung, and kidney diseases; the disclosed similarities of the rat, mouse, and human ACE2 structure and function; and the teaching in the specification that an ACE2 decreased state, such as cardiac, lung, and kidney disease, may be treated by administering to an animal in need thereof an effective amount of an agent that can increase the expression of ACE2; I believe that the currently claimed method of treatment could be practiced without undue experimentation in any mammal in need of such treatment. This is confirmed by the demonstration by Imai *et al.* that injecting ACE2 knockout mice or acid-treated wild-type mice with a rhACE2 protein protected the mice from severe acute lung injury; and the study in the piglet ARDS model showing that rhACE2 protein therapy stabilized or even decreased both pulmonary arterial pressure and systolic arterial pressure.

15. I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: 03-20-07

  
Nikolaus Neu, M.D.



## **EXHIBIT 1**

# CURRICULUM VITAE

Name: Nikolaus Neu  
Date of Birth: December 3rd, 1957 in Mittelberg, Austria  
Nationality: Austrian  
Marital status: Married, 2 Children (born 1985 and 1987)

## Education

1976.1982 University of Innsbruck, Medical School, Austria  
1979.1982 University of Innsbruck, Department of Psychology, Austria  
1979.1983 Teaching assistant University of Innsbruck, Medical School, Austria  
1981.1982 Research topics: - Characterization of MHC-specific antibodies  
- Immunogenetic analysis of autoimmune thyroiditis in animal models  
1982 Graduation from Medical School

## Postdoctoral Training and Employment History

1982.1985 Research Fellow at the University of Innsbruck, Medical School  
Research topics - Immunogenetic analysis of autoimmune thyroiditis  
- Production and characterization of monoclonal antibodies identifying specific lymphocyte subpopulations  
1985-1987 Postdoctoral fellow at the Department of Immunology and Infectious Diseases (Prof. Rose),  
The Johns Hopkins University, Baltimore, MD, USA.  
Faculty member at Johns Hopkins University since 1986.  
Research topic: Identification of mechanisms for autoimmunity using a mouse model of Coxsackievirus-induced myocarditis.  
1987-1991 Head of research group at the institute of pathology at the University of Innsbruck  
Research topic: - Mechanisms of autoimmunity in heart diseases  
- Clinical diagnosis of immune diseases  
1992 Habilitation in the field of „Functional pathology“

- 1991.1997    Pediatrician at the University Hospital Innsbruck  
 Main fields of interest:    Neonatology and intensive care  
    Hereditary immune diseases  
    Bronchoscopy
- Head of the research project „Mechanisms of Antigen Recognition and  
 therapeutic approaches in postinfectious Autoimmune Myocarditis“
- 1998                    Habilitation in the field of pediatry  
 Appointed professor and senior physician at the department for intensive care  
 in newborns, University Hospital Innsbruck
- since 2000    Head of the pediatric intensive care unit, University Hospital Innsbruck

#### **Miscellaneous**

- Winner of several scientific awards
- Keynote speaker at international meetings
- Reviewer for international journals
- Reviewer for scientific projects (Austrian Science Fund, EU-Projects, Israel Science Foundation)

# **PUBLIKATIONSLISTE**

## **Nikolaus Neu**

### **ORIGINALARBEITEN:**

1. Neu N, Hála K, Wick G: „Natural“ chicken antibodies to red blood cells are mainly directed against the B-G antigen, and their occurrence is independent of spontaneous autoimmune thyroiditis. *Immunogenetics* 1984; 19:269
2. Neu N, Hála K, Dietrich H, Wick G: Spontaneous autoimmune thyroiditis in Obese strain chickens: A genetic analysis of target organ abnormalities. *Clin. Immunol. Immunopathol.* 1985; 37:397
3. Krömer G, Schauenstein K, Neu N, Stricker K, Wick G: In vitro T cell hyperreactivity in Obese strain (OS) chickens is due to a defect in nonspecific suppressor mechanism(s). *J. Immunol.* 1985; 135:2458
4. Neu N, Hála K, Dietrich H, Wick G: Genetic background of spontaneous autoimmune thyroiditis in the Obese strain of chickens studied in hybrids with an inbred line. *Int. Archs. Allergy appl. Immun.* 1986; 80:168
5. Hála K, Schauenstein K, Neu N, Krömer G, Wolf H, Böck G, Wick G: A monoclonal antibody reacting with a membrane determinant expressed on activated chicken T lymphocytes. *Eur. J. Immunol.* 1986; 16:1331
6. Alvarez F, Neu N, Rose NR, Craig SW, Beisel KW: Heart-specific autoantibodies induced by Coxsackievirus B3: Identification of heart autoantigens. *Clin. Immunol. Immunopathol.* 1987; 43:129
7. Neu N, Beisel KW, Traystman MD, Rose NR, Craig SW: Autoantibodies specific for the cardiac myosin isoform are found in mice susceptible to Coxsackievirus B3-induced myocarditis. *J. Immunol.* 1987; 138:2488
8. Neu N, Craig SW, Rose NR, Beisel KW: Coxsackievirus-induced autoimmune myocarditis in mice: cardiac myosin autoantibodies do not cross-react with the virus. *Clin. Exp. Immunol.* 1987; 69:566
9. Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW: Cardiac myosin induces myocarditis in genetically predisposed mice. *J. Immunol.* 1987; 139:3630
10. Krömer G, Fässler R, Hála K, Böck G, Schauenstein K, Brezinschek HP, Neu N, Dietrich H, Jokober R, Wick G: Genetic analysis of extrathyroidal features of Obese strain (OS) chickens with spontaneous autoimmune thyroiditis. *Eur. J. Immunol.* 1988; 18:1499
11. Krömer G, Neu N, Kühr T, Dietrich H, Fässler R, Hála K, Wick G: Immunogenetic analysis of spontaneous autoimmune thyroiditis of Obese strain chickens. *Clin. Immunol. Immunopathol.* 1989; 52:202
12. Neu N, Plöier B, Öfner C: Cardiac myosin-induced myocarditis. Myosin autoantibodies are not involved in the induction of the disease. *J. Immunol.* 1990; 145:4094

13. Rabausch-Starz I, Neu N, Müller-Hermelink HK: Virusnachweis und pathologische Veränderungen in verschiedenen Phasen der Coxsackievirus B Myokarditis bei Mäusen. *Verh. Dtsch. Ges. Path.* 1990; 74:398
14. Neu N, Ploier B: Experimentally-induced autoimmune myocarditis: Production of heart myosin-specific autoantibodies within the inflammatory infiltrate. *Autoimmunity* 1991; 8:317
15. Pummerer CL, Berger P, Frühwirth M, Öfner C, Neu N: Cellular infiltrate, MHC antigen expression and immunopathogenic mechanisms in cardiac myosin-induced myocarditis. *Lab. Invest.* 1991; 65:538
16. Maczek C, Neu N, Wick G, Hála K: Target organ susceptibility and autoantibody production in an animal model of spontaneous autoimmune thyroiditis. *Autoimmunity* 1992; 12:277
17. Penninger JM, Neu N, Timms E, Wallace VA, Koh DR, Kishihara K, Pummerer CL, Mak TW: Induction of experimental autoimmune myocarditis in mice lacking CD4 or CD8 molecules. *J. Exp. Med.* 1993; 178:1837
18. Rabausch-Starz I, Schwaiger A, Grünewald K, Müller-Hermelink HK, Neu N: Persistence of virus and viral genome in myocardium after Coxsackievirus B3-induced murine myocarditis. *Clin. Exp. Immunol.* 1994; 96:69
19. Unsinn KM, Neu N, Krejci A, Posch A, Menardi G, Gaßner I: Pallister-Hall syndrome and McKusick-Kaufmann syndrome: ohne entity? *J. Med. Genet.* 1995; 32:125
20. Bachmaier K, Mair J, Offner F, Pummerer CL, Neu N: Serum cardiac troponin T and creatine kinase-MB elevations in murine autoimmune myocarditis. *Circulation* 1995; 92:1927
21. Pummerer CL, Grässl G, Sailer M, Bachmaier KW, Penninger JM, Neu N: Cardiac myosin-induced myocarditis: Target recognition by autoreactive T cells requires prior activation of cardiac interstitial cells. *Lab. Invest.* 1996; 74:845
22. Pummerer CL, Luze K, Grässl G, Bachmaier K, Offner F, Burrell SK, Lenz DM, Zamborelli TJ, Penninger JM, Neu N: Identification of cardiac myosin peptides capable of inducing autoimmune myocarditis in BALB/c mice. *J. Clin. Invest.* 1996; 97:2057
23. Bachmaier K, Pummerer CL, Shahinian A, Ionescu J, Neu N, Mak TW, Penninger JM: Induction of autoimmunity in the absence of CD28 costimulation. *J. Immunol.* 1996; 157:1752
24. Bachmaier K, Pummerer CL, Kozieradzki I, Pfeffer K, Mak T, Neu N, Penninger JM: Low-molecular-weight tumor necrosis factor receptor p55 controls induction of autoimmune heart disease. *Circulation* 1997; 95:655
25. Grässl G, Pummerer CL, Horak I, Neu N: Induction of autoimmune myocarditis in Interleukin-2 – deficient mice. *Circulation* 1997; 95:1773
26. Bachmaier K, Neu N, Pummerer C, Duncan GS, Mak TW, Matsuyama T, Penninger JM: iNOS expression and nitrotyrosine formation in the myocardium in response to

inflammation is controlled by the interferon regulatory transcription factor 1. *Circulation* 1997; 96:585-91

27. Heitger A, Neu N, Kern H, Panzer-Grümayer ER, Greinix H, Nachbaur D, Niederwieser D, Fink FM: Essential role of the thymus to reconstitute naive (CD45RA<sup>+</sup>) T helper cells after human allogeneic bone marrow transplantation. *Blood* 1997; 90:850
28. Heitger A, Maurer K, Neu N, Fink FM: Capillary leak syndrome in a patient with septicemia and granulocyte-colony-stimulating factor (G-CSF)-induced accelerated granulopoiesis. *Med. Pediatr. Oncol.* 1998; 31:126
29. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM: Chlamydia infections and heart disease linked through antigenic mimicry. *Science* 1999; 283:1335
30. Bachmaier K, Neu N, Yeung RS, Mak TW, Liu P, Penninger JM. Generation of humanized mice susceptible to peptide-induced inflammatory heart disease. *Circulation*. 1999; 99:1885
31. Janecke AR, Unsinn K, Kreczy A, Baldissera I, Gassner I, Neu N, Utermann G, Muller T: Adducted thumb-club foot syndrome in sibs of a consanguineous Austrian family. *J. Med. Genet.* 2001; 38:265
32. Frühwirth M, Clodi K, Heitger A, Neu N: Lymphocyte diversity in a 9-year-old boy with idiopathic CD4<sup>+</sup> T cell lymphocytopenia. *Int. Arch. Allergy Immunol.* 2001; 125:80
33. Geiger R, Pajk W, Neu N, Maier S, Kleinsasser A, Fratz S, Navarro-Psiha S, Fischer V, Trembl B, Loeckinger A: Tezosentan decreases pulmonary artery pressure and improves survival rate in an animal model of meconium aspiration. *Pediatr. Res.* 2006; 59:147

## ÜBERSICHTSARBEITEN:

1. Wick G, Möst J, Schauenstein K, Krömer G, Dietrich H, Ziemiecki A, Fässler R, Schwarz S, Neu N, Hala K. Spontaneous autoimmune thyroiditis – a bird's eye view. *Immunology Today* 1985; 6:359
2. Wick G, Krömer G, Neu N, Fässler R, Ziemiecki A, Müller RG, Ginzel M, Beladi I, Kühr T, Hala K: The multi-factorial pathogenesis of autoimmune disease. *Immunology Letters* 1987; 16:249
3. Rose NR, Herskowitz A, Neumann DA, Neu N: Autoimmune myocarditis: a paradigm of post-infection autoimmune disease. *Immunology Today* 1988; 9:117
4. Krömer G, Gastinel LN, Neu N, Auffray Ch, Wick G: How many genes code for organ-specific autoimmunity? *Autoimmunity* 1990; 6:215
5. Neu N, Klieber R, Frühwirth M, Berger P: Cardiac myosin-induced myocarditis as a model for post-infectious autoimmunity. *Eur. Heart J.* 1991; 12:117
6. Neu N, Pummerer CL, Rieker T, Berger P: T cells in cardiac myosin-induced myocarditis. *Clin. Immunol. Immunopathol.* 1993; 68:107
7. Pummerer CL, Grässl G, Neu N: Cellular mechanisms in myosin-induced myocarditis.

E. Heart J. 1995; 16:71

8. Penninger JM, Neu N, Bachmaier K: A genetic map of autoimmune heart disease. *The Immunologist* 1996; 4:131
9. Penninger JM, Liu P, Pummerer CL, Neu N, Bachmaier K: Cellular and molecular mechanisms in murine autoimmune myocarditis. *APMIS* 1997; 105:13

### **BUCHBEITRÄGE:**

1. Rose NR, Beisel KW, Herskowitz A, Neu N, Wolfgram LJ, Alvarez F, Traystman MD, Craig SW: Cardiac myosin and autoimmune myocarditis. In: *Autoimmunity and Autoimmune disease*. D. Everet (Ed.), CIBA Foundation Symposium 129, Wiley, Chichester 1986; pp 3-24
2. Kuppers RC, Neu N, Rose NR. Animal models of autoimmune thyroid disease. Farid NR (Ed.) Liss AR, Inc., New York 1988; pp 111-131
3. Rose NR, Neu N, Neumann DA, Herskowitz A: Myocarditis: A postinfectious autoimmune disease. In: *New Concepts in Viral Heart Disease*. Schultheiß HP (Ed), Springer Verlag Berlin-Heidelberg 1988; pp 139-147
4. Neu N, Krömer G, Ploier B, Rose NR: Is Coxsackievirus B3-induced myocarditis in A/J mice mediated by an autoimmune response to cardiac myosin? In: *New Concepts in Viral Heart Disease*, Schultheiß HP (Ed.), Springer Verlag-Heidelberg 1988; pp 160-167
5. Holter W, Neu N: Morphologie und Funktion des spezifischen Immunsystems. In: *Pädiatrische Hämatologie und Onkologie*, Gadner, Gaedike, Miemeyer, Ritter (Ed.), Springer Medizin Verlag Heidelberg 2006; pp 237-245

## **EXHIBIT 2**



## Physiological roles of angiotensin-converting enzyme 2

U. Danilczyk<sup>a,b</sup>, U. Eriksson<sup>c</sup>, G. Y. Oudit<sup>d</sup> and J. M. Penninger<sup>a,b,\*</sup>

<sup>a</sup> Departments of Medical Biophysics and Immunology, University of Toronto, 620 University Avenue, Toronto M5G 2C1, Ontario (Canada)

<sup>b</sup> IMBA, Institute for Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr Gasse 7, 1030 Vienna (Austria), Fax: +43 1 79730 459, e-mail: josef.penninger@imba.oew.ac.at

<sup>c</sup> Departments of Research and Internal Medicine, Basel University Hospital, 4031 Basel (Switzerland)

<sup>d</sup> Faculty of Medicine, Heart and Stroke/Richard Lewar Centre of Excellence, Fitzgerald Building, University of Toronto, Rm 68, 150 College St., Toronto M5S 2E3, Ontario (Canada)

**Abstract.** Angiotensin-converting enzyme 2 (ACE2) is a recently discovered homologue of the key enzyme of the renin-angiotensin system, the angiotensin-converting enzyme. The ACE2 enzyme is mainly expressed in cardiac blood vessels and tubular epithelia of the kidneys. Together with ACE2's unique metallopeptidase activity, the restricted tissue distribution suggests a distinctive physiological function in blood pressure, blood flow and fluid regulation. The *ace2* gene was mapped to quantitative trait loci affecting susceptibility to hypertension

in rats. Furthermore, ACE2 appears to be a negative regulator of ACE in the heart. ACE2 messenger RNA and protein levels are substantially regulated in the kidney of diabetic and pregnant rats. The mechanism of ACE2 function and its physiologic significance are not yet fully understood; however, as ACE2 differs in its specificity and physiological role from ACE, this opens a new potential venue for drug discovery aimed at cardiovascular disease, hypertension and diabetic complications.

**Key words.** Angiotensin-converting enzyme 2; knockout mice; renin-angiotensin system.

### Introduction

For the last 50 years, angiotensin-converting enzyme (ACE) has assumed a central position in the renin-angiotensin system (RAS). The RAS is a major regulatory network that maintains blood pressure, fluid and electrolyte balance and electrolyte homeostasis. ACE functions primarily as a 'peptidyl dipeptidase', removing dipeptides from the C-terminus of peptide substrates [1]. Its primary substrate was identified as angiotensin I. ACE processes the decapeptide angiotensin I to the eight-amino-acid peptide angiotensin II, which functions as a strong vasoconstrictor. In parallel, ACE also inactivates the vasodilator peptides bradykinin and kallidin, and thus potentiates the vasopressor response mediated by angiotensin II [1]. Inhibition of ACE's enzymatic activity has a powerful effect in reduction of blood pressure; thus

small molecule inhibitors of human endothelial ACE are used for antihypertensive therapies [2]. In addition to their effectiveness in treating hypertension, ACE inhibitors have been found to lower the risk of coronary heart disease and stroke. Furthermore, they improve the prognosis of patients with cardiac failure and diabetic nephropathy (for review, see [3]).

With the discovery of ACE2/ACEH by Donoghue [4] and Tipins [5], a new level of complexity was added to the RAS. The ACE2 gene is located in the region of the X chromosome (Xp22), which maps to quantitative trait loci (QTL) in hypertensive rats [6–8]. Consistent with a possible role in cardiorenal function, ACE2 was found to be predominantly expressed in endothelia of the heart and in tubular epithelia of the kidney [4, 5]. In humans, ACE2 was also found in the gastrointestinal tract [9]. Additionally, in mouse, ACE2 has been detected in lungs [10]. While ACE and ACE2 protein are similar in their metalloprotease catalytic domains, they differ in their substrate specificity [11]. Analysis of ACE2 expression and the

\* Corresponding author.

physiological role of its substrates suggest that ACE2 may act as a tissue-specific negative feedback regulator of the RAS [3]. Furthermore, the differences observed in phenotype between the genetically engineered *ace* and *ace2* mice [12] all suggest a role for ACE2 in heart pathophysiology. Moreover, since it has been shown that ACE2 acts not only on the angiotensin I and angiotensin II peptides, but also efficiently cleaves the C-terminal residues from several other peptides such as apelin-13 and dynorphin A 1–13, unrelated to angiotensin I [4], ACE2 function may not be limited only to RAS.

### Substrate specificity of ACE2

In the classic pathway of RAS, angiotensin I is generated from the circulating precursor angiotensinogen by the action of renin, an enzyme secreted from juxtaglomerular cells at the renal afferent arterioles [13]. Angiotensin I has little effect on blood pressure and is converted by ACE to angiotensin II. Angiotensin II, a potent vasopressor, acts on the blood vessels and the kidneys by binding to the G-protein-coupled receptors AT<sub>1</sub> and AT<sub>2</sub>. In contrast, ACE2 cleaves the C-terminal amino acid of angiotensin I to a nonapeptide angiotensin 1–9 [4]. In rat and human plasma angiotensin 1–9 levels are twice those of angiotensin II [14, 15], and angiotensin 1–9 accumulates in animals treated with ACE inhibitors [16]. The biological function of angiotensin 1–9 is still not well defined. However, angiotensin 1–9 is thought to potentiate angiotensin II-mediated vasoconstriction in isolated rat aortic rings and to have pressor effects in awake rats [17].

Angiotensin 1–9 was also shown to have weak pressor effects in anesthetized rats and dogs, and vasoconstricting activity in isolated rat aorta [17].

ACE2 directly converts angiotensin II to angiotensin 1–7 [4, 18]. In animals, angiotensin 1–7 has been proposed to be an important regulator of cardiovascular function, promoting vasodilatation, apoptosis and growth arrest [19, 20]. However, its functional significance in humans is still controversial. Aside from the degradation of the vasoconstrictor angiotensin II, the formation of the vasodilatory angiotensin 1–7 might reflect the negative regulatory function of ACE2 in the presence of an activated RAS.

In addition to its activity as angiotensin-converting enzyme, ACE2 can remove in *in vitro* assays the C-terminal residue from other vasoactive peptides, including neurotensin, kinetensin (a neurotensin-related peptide) and des-Arg bradykinin (fig. 1). The kinin metabolites, des-Arg<sup>10</sup>-kallidin (des-Arg<sup>10</sup>-Lys<sup>1</sup>-bradykinin) and des-Arg<sup>9</sup>-bradykinin activate the G-protein-coupled B<sub>1</sub> receptor [21], which is upregulated in response to tissue injury and may be important in mediating inflammatory responses. Furthermore, ACE2 also acts on apelin-13 and apelin-36 peptides with high catalytic efficiency [18]. These two forms of apelin were recently identified as endogenous ligands for the human APJ receptor, which is a homolog of the angiotensin receptor AT<sub>1</sub> [22]. The role of the apelins is not fully understood. Whereas systemic administration of apelin-13 promotes hypotension in rats [23], it has been shown that apelin-13 promotes vasoconstriction in endothelium-denuded coronary arteries [23]. Intraperitoneal injection of apelin-13 in rats increases water intake [23]. Two opioid peptides, dynorphin A 1–13 and

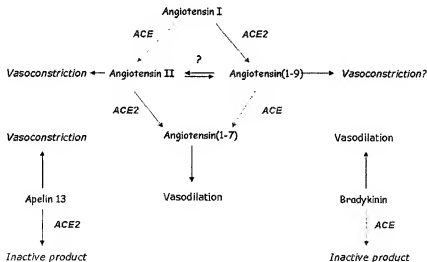


Figure 1. Hypothetical model of ACE and ACE2 functions. Angiotensin I serves as a substrate for both ACE and ACE2. Angiotensin II is known to act as vasoconstrictor *in vivo*. The function of Angiotensin 1–9 is still not well understood. Both ACE and ACE2 are involved in the production of the vasodilator peptide angiotensin 1–7. From genetic experiments it appears that ACE and ACE2 have complementary functions by negatively regulating each other in the RAS.

Table 1. ACE2 functions as a carboxymonopeptidase with a preference for C-terminal hydrophobic or basic residues. The ACE2 substrates, the amino acids cleaved by ACE2 (underline) and the receptors of some of the ACE2 substrates are indicated. The physiological functions are not always well defined.

ACE2 substrates/products	Receptor	Physiological functions of ACE2 substrate/product
Angiotensin I <i>Asp Arg Val Thr Ile His Pro Phe His Leu</i>		unknown/vasoconstrictor?
Angiotensin II <i>Asp Arg Val Tyr Ile His Pro Phe</i>	G-protein-coupled receptors AT <sub>1</sub> and AT <sub>2</sub>	vasoconstrictor/vasodilator
Apelin-36 <i>c-term-Gln Arg Pro Arg Leu Ser His Lys Gly Pro Met Pro Phe</i>	APJ receptor (homolog of AT <sub>1</sub> )	vasoconstriction, vasodilation, water intake/inactive product
Apelin-13 <i>Gln Arg Pro Arg Leu Ser His Lys Gly Pro Met Pro Phe</i>		
[Des-Arg] <sup>1</sup> Bradykinin <i>Arg Pro Gly Phe Ser Pro Phe</i>	G-protein-coupled receptors B <sub>1</sub>	tissue injury/inflammatory responses/inactive product
Lys [Des-Arg] <sup>1</sup> Bradykinin <i>Lys Arg Pro Pro Gly Phe Ser Pro Phe</i>		
Neurotensin <i>pGlu-Leu Tyr Gly Asn Lys Pro Arg</i>	kappa and delta G-protein-coupled opioid receptor	pain perception
Dynorphin A <i>Tyr Gly Gly Phe Leu Arg Arg Ile Arg Pro Lys Leu Lys</i>		
B casamorphin <i>Tyr Pro Phe Val Glu Pro Ile</i>		

$\beta$ -casamorphin are also substrates of ACE2 [18] (table 1). These peptides activate kappa and delta G-protein opioid receptors that regulate pain perception and among other functions may have negative effects on cardiomyocyte contractility [24]. ACE2, however, failed to cleave bradykinin and 15 other unrelated vasoactive and hormonal peptides [4]. Although the biological peptides angiotensin II, apelin-13, dynorphin A 1–13 and des-Arg<sup>1</sup>-bradykinin are good ACE2 substrates *in vitro*, their role as physiological substrates of ACE2 is still unclear.

### ACE2 and blood pressure

Based on the potential *in vivo* functions of angiotensin 1–9, angiotensin 1–7 and des-Arg bradykinin, it is tempting to speculate that ACE2 plays a role in the regulation of blood pressure homeostasis (fig. 1). Indeed, the *ace2* gene is located in the region of the X chromosome (Xp22), which maps to a QTL in the Sabra, SHR and SHRSP rat models of hypertension [6–8, 25]. These QTLs carry a significant logarithm-of-the-odds score that suggests the presence of a hypertension-related gene within the chromosomal span demarcated by the QTLs. In the Sabra rat model of salt-sensitive hypertension, ACE2 messenger RNA (mRNA) and protein levels are diminished in the hypertension-prone SBH/y strain when compared with the hypertension-resistant SBN/y strain [6]. Baseline systolic blood pressure is 10–20 mm Hg higher in the Sabra hypertension-prone SBH/y strain than in hypertension-resistant SBN/y rats. [7] Also, during salt

loading, ACE2 levels are diminished even further in SBH/y animals, which become overtly hypertensive, whereas the levels remain unchanged in SBN/y rats, which remain normotensive [6, 7]. Spontaneously hypertensive rats (SHR) and spontaneously hypertensive stroke-prone rats (SHRSP) develop hypertension without an apparent external hypertensive stimulus. ACE2 expression is consistently lower in both hypertensive SHR and SHRSP strains compared to the normotensive Wistar Kyoto (WKY) control rats [26]. In all of these rat models of hypertension, ACE2 mRNA and protein levels were greatly reduced in the kidneys in association with increased blood pressure [6].

These genetic data together with the *in vitro* biochemical data imply a pathophysiologic role of ACE2 in essential hypertension. It is thought that a reduction in ACE2 levels results in impaired degradation of angiotensin II, and reduced formation of vasodilator by-products on the level of the kidney endothelium, thus promoting a blood pressure increase [26]. However, the *in vivo* studies of two recent knockout mice report conflicting results. Crackower et al. report that *ace2* null mice do not show increased blood pressure compared with control littermates, despite increased angiotensin II plasma and tissue levels [6]. In fact, at 6 months of age, male *ace2* knockout mice had reduced blood pressure as measured using the tail-cuff technique in conscious restrained mice [6]. These results were confirmed using invasive hemodynamic measurement in anesthetized *ace2* null mice that showed reduced systolic blood pressure and mean blood pressure as compared with littermate controls [27]. However, since this

reduction in blood pressure coincided with impaired cardiac function, it is difficult to separate these independent effects on systemic vascular response in *ace2* null mice. In contrast, Allred et al. [28] reported slightly elevated baseline blood pressure levels in a second knockout mouse line lacking the *ace2* gene. These *ace2* null mice also showed a significantly enhanced vasopressor response upon angiotensin II infusion compared to wild-type controls. The higher baseline blood pressure in *ace2*-deficient mice is consistent with the findings in the Sabra model, in which SBH/y with the lower ACE2 expression display higher baseline blood pressure [26]. However, blocking ACE2 by the strong peptide inhibitor DX512 in spontaneously hypertensive rats results in a dose-dependent blood pressure decrease and reflex tachycardia with the maximal average depressor response at  $70.5 \pm 4.6$  mm Hg from an average mean arterial pressure of  $155 \pm 10$  mm Hg at baseline [17]. This in vivo demonstration of the antihypertensive effect of an ACE2 inhibitor contradicts Allred's observation of increased blood pressure in *ace2* null mice. However, since essential hypertension depends on the concerted contribution of multiple genetic and environmental factors, the conflicting data from in vivo studies with ACE2 antagonists and *ace2* null mice might reflect effects of genetic background, age, gender and experimental setup [27]. Taken together, it still remains unclear what the net effect is of the interplay between angiotensin II and the ACE2-mediated peptides angiotensin 1–7 and angiotensin 1–9. It has to be clarified whether, in the relative absence of ACE2, an angiotensin II effect predominates, leading to vasoconstriction and hypertension, or whether compensating mechanisms maintain normal or lower blood pressure dependent on defined genetic backgrounds. The mechanism that regulates blood pressure through the production of angiotensin II was thought to be well understood, but given the complexity of the systems involved, additional studies on mutant mice and specific blocking agents are needed to further our understanding of the physiologic role of ACE2 in blood pressure regulation.

### Loss of ACE2 impairs heart function

Experiments with inhibitors of ACE and angiotensin II receptors suggest the involvement of the RAS in the regulation of heart function and cardiac hypertrophy. However, neither *ace* [29,30] nor *angiotensinogen* [31] deficient mice show defects in heart development or are prone to heart disease. In contrast, *ace2* deficient mice exhibit a reduction in cardiac contractility and a significant decrease in aortic and ventricular pressure [6]. Therefore, ACE2 appears to be an important regulator of heart function in vivo. The observed phenotype closely resembles cardiac stunning/hibernation in human and animal models [32]. Cardiac stunning and hibernation re-

flect adaptive responses to prolonged tissue hypoxia that occurs in coronary artery disease or following bypass surgery [33]. Accordingly, the hearts of *ace2* null mice show upregulation of mRNA expression of hypoxia-inducible genes such as *BNIP3* [34] and *PAI-1* [35]. The magnitude of increased expression of these hypoxia-inducible genes resembles previously observed levels in other hypoxic models, such as myocyte-specific vascular endothelial growth factor mutant mice [36].

Interestingly, ablation of ACE expression on an *ace2* mutant background completely abolished the cardiac dysfunction phenotype of *ace2* single knockout mice [6]. In fact, the heart function of *ace/ace2* double mutant mice was similar to *ace* single mutant and wild-type littermates. The normal cardiac functions of *ace/ace2* double mutant mice suggest that an ACE product, most likely angiotensin II, accounts for the observed cardiac dysfunction of *ace2* single mutant mice. In fact, cardiac myocytes express angiotensin II receptors and undergo hypertrophy in response to angiotensin II. Taken together, it is intriguing to speculate that an excess in the vascular tone of *ace2* null hearts due to unopposed angiotensin II-mediated effects is responsible for the observed heart phenotype.

### Renal function of ACE2

In the kidneys the local RAS plays a significant role in the control of organ function and blood pressure regulation. Reduction in systemic blood pressure, decrease in extracellular volume and pathophysiologic conditions affecting the renal arteries all result in reduced glomerular filtration and decreased amounts of sodium entering the proximal tubuli. As a result, renin secretion is stimulated in the kidneys. This mechanism, termed tubuloglomerular feedback (see [37] for review) ultimately results in increased angiotensin II and aldosterone production, counterbalancing reduced blood pressure and/or a decreased extracellular volume.

Within the kidney, ACE2 has a distribution similar to ACE. ACE2 is present in distal tubules and to a much lesser extent in glomeruli, as assessed by both gene and protein expression [38]. ACE2 levels are reduced in experimental diabetic nephropathy [38]. In the context of essential hypertension, previous studies demonstrated that the ACE2 product angiotensin 1–7 counteracting the pressor, trophic and antidiuretic actions of angiotensin II was elevated in untreated essential hypertensive subjects [39]. In pregnant rats angiotensin 1–7 levels are increased in association with increased ACE2 expression, suggesting that ACE2 may contribute to the local production and overexpression of angiotensin 1–7 in the kidney [40]. Taken together, these findings suggest that angiotensin 1–7 might be a critical link in mediating the negative regulatory feedback between ACE and ACE2. From a more general point of view, it is possible that the

relative balance of vasoconstrictor and vasodilatory angiotensin peptides modulates both hemodynamic and trophic effects within the kidney. Nevertheless, the physiological role of ACE2 remains to be determined. Of note, *ace2* deficient mice have normal renal medullary development and normal renal architecture [6]. So far, it is not known whether *ace2* null mice exhibit functional alterations in terms of altered tubuloglomerular feedback mechanism, urine concentration or electrolyte balance.

### Concluding remarks

Almost 50 years after the discovery of ACE, our understanding of the pathways contributing to the formation of biologically active forms of angiotensinogen peptides are challenged with the discovery of ACE2. It has become apparent that additional intermediates are involved in a feedback regulation of the RAS. Furthermore, ACE2 was shown to function not only in the metabolism of angiotensin I but also in the catalysis of opioid peptides, apelin, neurotensin and kinetins. In addition, ACE2 has gained recognition as an important regulatory enzyme in blood vessels supplying the heart and in the arterioles and tubules of the kidneys. High blood pressure is a major risk factor for myocardial infarction, cerebrovascular disease and stroke. The elucidation of the physiological role of ACE2 and the characterization of ACE2 substrates and its products may ultimately lead to the development of new therapeutics against hypertension and heart failure.

**Acknowledgements.** The IMBA funds research in the authors' laboratories. U.E. acknowledges support from the Gottfried and Julia Bangerter Foundation and the Department of Internal Medicine, Basel University Hospital. We thank M.J. Crackower, R. Sarao, P. Backx and many others for their contributions.

- 1 Skeggs L. T. (1956) The preparation and function of the hypertension converting enzyme. *J. Exp. Med.* 103: 295–299
- 2 Cushman D. W. and Ondetto M. A. (1980) Inhibitors of angiotensin converting enzyme for treatment of hypertension. *Biochem. Pharmacol.* 29: 1871–1877
- 3 Turner A. J. and Hooper N. M. (2002) The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol. Sci.* 23: 177–183
- 4 Donoghue M., Hsieh F., Baronas E., Godbout K., Gosselin M., Stagliano N. et al. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. *Circ. Res.* 87: E1–E9
- 5 Tipnis S. R., Hooper N. M., Hyde R., Karran E., Christie G. and Turner A. J. (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J. Biol. Chem.* 275: 33238–33243
- 6 Crackower M. A., Sarao R., Oudit G. Y., Yagil C., Kozieradzki I., Scanga S. E. et al. (2002) Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822–828
- 7 Yagil C., Katni G., Rubattu S., Stolpe C., Kreutz R., Lindpaintner K. et al. (1996) Development, genotype and phenotype of a new colony of the Sabra hypertension prone (SBH/y) and resistant (SBN/y) rat model of salt sensitivity and resistance. *J. Hypertens.* 14: 1175–1182
- 8 Yagil C., Sapojnikov M., Kreutz R., Zurcher H., Ganten D. and Yagil Y. (1999) Role of chromosome X in the Sabra rat model of salt-sensitive hypertension. *Hypertension* 33: 261–265
- 9 Harner D., Gilbert M., Bornan R. and Clark K.L. (2002) Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett.* 532: 107–110
- 10 Komatsu T., Suzuki Y., Imai J., Sugano S., Hida M., Tanigami A. et al. (2002) Molecular cloning, mRNA expression and chromosomal localization of mouse angiotensin-converting enzyme-related carboxypeptidase (mACE2). *FEBS Lett.* 521: 217–220
- 11 Eriksson U., Danilczyk U. and Penninger J. M. (2002) Just the beginning: novel functions for angiotensin-converting enzymes. *Curr. Biol.* 12: R745–R752
- 12 Danilczyk U., Eriksson U., Crackower M. A. and Penninger J. M. (2003) A story of two ACEs. *J. Mol. Med.* 81: 227–234
- 13 Inagami T. (1994) The renin angiotensin system. *Essays Biochem.* 28: 147–164
- 14 Johnson H., Kourits S., Waters J. and Drummer O. H. (1989) Radioimmunoassay for immunoreactive [des-Leu(10)]-angiotensin I peptides (Elmsford) 10: 489–492
- 15 Oparil S., Tregear G. W., Koerner T., Barnes B. A. and Haber E. (1971) Mechanism of pulmonary conversion of angiotensin I to angiotensin II in the dog. *Circ. Res.* 29: 682–690
- 16 Drummer O. H., Kourits S. and Johnson H. (1990) Effect of chronic enalapril treatment on enzymes responsible for the metabolism of angiotensin I and formation of angiotensin II. *Biochem. Pharmacol.* 39: 513–518
- 17 Huang L., Sexton D. L., Skogerson K., Devlin M., Smith R., Sanyal I. et al. (2003) Novel peptide inhibitors of angiotensin-converting enzyme 2. *J. Biol. Chem.* 278: 15532–15540
- 18 Vickers C., Hales P., Kaushik V., Dick L., Gavin J., Tang J. et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.* 277: 14838–14843
- 19 Iyer S. N., Averill D. B., Chappell M. C., Yamada K., Allred A. J. and Ferrario C. M. (2000) Contribution of angiotensin-(1-7) to blood pressure regulation in salt-depleted hypertensive rats. *Hypertension* 36: 417–422
- 20 Ren Y., Garvin J. L. and Carretero O. A. (2002) Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39: 799–802
- 21 Duka I., Kintsurslavskii E., Gavras I., Johns C., Bresnahan M. and Gavras H. (2001) Vasoactive potential of the B(1) bradykinin receptor in normotension and hypertension. *Circ. Res.* 88: 275–281
- 22 Hosoya M., Kawamata Y., Fukusumi S., Fujii R., Habata Y., Hinuma S. et al. (2000) Molecular and functional characteristics of APJ: tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J. Biol. Chem.* 275: 21061–21067
- 23 Tatemoto K., Takayama K., Zou M. X., Kumaki I., Zhang W., Kumano K. et al. (2001) The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul. Pept.* 99: 87–92
- 24 Ventura C., Spurgeon H., Lakatta E. G., Guarnieri C. and Capogrossi M. C. (1992) Kappa and delta opioid receptor stimulation affects cardiac myocyte function and Ca<sup>2+</sup> release from an intracellular pool in myocytes and neurons. *Circ. Res.* 70: 66–81
- 25 Hilbert P., Lindpaintner K., Beckmann J. S., Serikawa T., Soubrier F., Dubay C. et al. (1991) Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. *Nature* 353: 521–529
- 26 Yagil Y. and Yagil C. (2003) Hypothesis: ACE2 modulates blood pressure in the mammalian organism. *Hypertension* 41: 871–873

- 27 Oudit G. Y., Crackower M. A., Backx P. H. and Penninger J. M. (2003) The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc. Med.* **13**: 93–101
- 28 Allred A. J., Donoghue M., Acton S. and Coffman T. M. (2002) Regulation of blood pressure by the angiotensin converting enzyme homologue ACE2. Paper presented at 35th Annual Meeting of the American Society of Nephrology, 1–4 November 2002, Philadelphia, PA. Abstract available at <http://www.abstracts-on-line.com/abstracts/asn/ao1.asp>
- 29 Krege J. H., John S. W., Langenbach L. L., Hodgin J. B., Haggman J. R., Bachman E. S. et al. (1995) Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* **375**: 146–148
- 30 Esther C. R. Jr, Howard T. E., Marino E. M., Goddard J. M., Capecci M. R. and Bernstein K. E. (1996) Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology and reduced male fertility. *Lab. Invest.* **74**: 953–965
- 31 Tanimoto K., Sugiyama F., Goto Y., Ishida J., Takimoto E., Yagami K. et al. (1994) Angiotensinogen-deficient mice with hypotension. *J. Biol. Chem.* **269**: 31334–31337
- 32 Murphy A. M., Kogler H., Georgakopoulos D., McDonough J. L., Kass D. A., Van Eyk J. E. et al. (2000) Transgenic mouse model of stunned myocardium. *Science* **287**: 488–491
- 33 Heusch G. (1998) Hibernating myocardium. *Physiol. Rev.* **78**: 1055–1085
- 34 Sowter H. M., Ratcliffe P. J., Watson P., Greenberg A. H. and Harris A. L. (2001) HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* **61**: 6669–6673
- 35 Kietzmann T., Roth U. and Jungermann K. (1999) Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. *Blood* **94**: 4177–4185
- 36 Giordano F. J., Gerber H. P., Williams S. P., VanBruggen N., Bunting S., Ruiz-Lozano P. et al. (2001) A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. *Proc. Natl. Acad. Sci. USA* **98**: 5780–5785
- 37 Kurokawa K. (1998) Tubuloglomerular feedback: its physiological and pathophysiological significance. *Kidney Int. Suppl.* **67**: S71–S74
- 38 Tikellis C., Johnston C. I., Forbes J. M., Burns W. C., Burrell L. M., Risvanis J. et al. (2003) Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* **41**: 392–397
- 39 Ferrario C. M., Martelli N., Yunis C., Flack J. M., Chappell M. C., Brosnihan K. B. et al. (1998) Characterization of angiotensin-(1–7) in the urine of normal and essential hypertensive subjects. *Am. J. Hypertens.* **11**: 137–146
- 40 Brosnihan K. B., Neves L. A., Joyner J., Averill D. B., Chappell M. C., Sarao R. et al. (2003) Enhanced renal immunocytochemical expression of ANG-(1–7) and ACE2 during pregnancy. *Hypertension* (Epub ahead of print)



To access this journal online:  
<http://www.birkhauser.ch>

## **EXHIBIT 3**

# Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®



*Learn and Live.™*

## **Angiotensin-Converting Enzyme II in the Heart and the Kidney**

Ursula Danilczyk and Josef M. Penninger

*Circ. Res.* 2006;98:463-471

DOI: 10.1161/01.RES.0000205761.22353.5f

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2006 American Heart Association. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:  
<http://circres.ahajournals.org/cgi/content/full/98/4/463>

Subscriptions: Information about subscribing to Circulation Research is online at  
<http://circres.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore, MD 21202-2436. Phone 410-5280-4050. Fax: 410-528-8550. Email: [journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at  
<http://www.lww.com/static/html/reprints.html>



This Review is part of a thematic series on **Angiotensin Converting Enzyme**, which includes the following articles:  
Six **Truisms** Concerning ACE and the Renin-Angiotensin System Educated from the Genetic Analysis of Mice

ACE II in the Heart and the Kidney

Signaling by the Angiotensin Converting Enzyme  
ACE Polymorphisms  
ACE and Vascular Remodeling

Kathy K. Griendling and Rudi Busse, Editors

## Angiotensin-Converting Enzyme II in the Heart and the Kidney

Ursula Danilczyk, Josef M. Penninger

**Abstract**—The renin-angiotensin system (RAS) has been recognized for many years as critical pathway for blood pressure control and kidney functions. Although most of the well-known cardiovascular and renal effects of RAS are attributed to angiotensin-converting enzyme (ACE), much less is known about the function of ACE2. Experiments using genetically modified mice and inhibitor studies have shown that ACE2 counterbalances the functions of ACE and that the balance between these two proteases determines local and systemic levels of RAS peptides such as angiotensin II and angiotensin(1–7). *Ace2* mutant mice exhibit progressive impairment of heart contractility at advanced ages, a phenotype that can be reverted by loss of ACE, suggesting that these enzymes directly control heart function. Moreover, ACE2 is also found to be upregulated in failing hearts. In the kidney, ACE2 protein levels are significantly decreased in hypertensive rats, suggesting a negative regulatory role of ACE2 in blood pressure control. Moreover, ACE2 expression is downregulated in the kidneys of diabetic and pregnant rats and ACE2 mutant mice develop late onset glomerulonephritis resembling diabetic nephropathy. Importantly, ACE2 not only controls angiotensin II levels but functions as a protease on additional molecular targets that could contribute to the observed *in vivo* phenotypes of ACE2 mutant mice. Thus, ACE2 seems to be a molecule that has protective roles in heart and kidney. The development of drugs that could activate ACE2 function would allow extending our treatment options in diabetic nephropathy, heart failure, or hypertension. (*Circ Res*. 2006;98:463–471.)

**Key Words:** angiotensin-converting enzyme 2 ■ knockout mice ■ renin-angiotensin system

The renin-angiotensin system (RAS) has been studied for more than a century. Angiotensin II (Ang II), its main active peptide, exerts a plethora of effects on several target organs, including blood vessels, kidney, and heart, and influences many physiological functions, such as blood pressure, fluid and electrolyte balance, and electrolyte homeostasis.<sup>1</sup> In animal models, administration of exogenous Ang II, in addition to its effect on blood pressure, is known to cause

neurotic cardiac, arterial, and renal lesions,<sup>2</sup> inhibit fibrinolysis,<sup>3</sup> stimulate formation of reactive oxygen species,<sup>4</sup> and induce apoptosis.<sup>5</sup> Endogenous Ang II excess plays a key role in congestive heart failure and ischemic heart disease.<sup>6,7</sup> Although the role of Ang II in various physiological and pathophysiological processes has been studied in numerous systems, assessment of how endogenous levels of Ang II are regulated by the opposing action of two carboxypeptidases,

Original received September 16, 2005; resubmission received November 28, 2005; revised resubmission received December 22, 2005; accepted January 13, 2006.

From the IMBA, Institute for Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria.

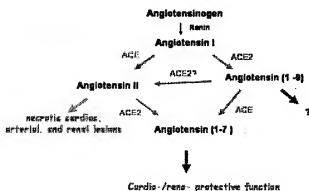
J.M.P. has shares in a company that develops recombinant human ACE2 for treatment of acute lung failure.

Correspondence to J.M. Penninger, IMBA, Institute for Molecular Biotechnology of the Austrian Academy of Sciences, D. Bohr Gasse 7, A-1030 Vienna, Austria. E-mail: josef.penninger@imba.oeaw.ac.at

© 2006 American Heart Association, Inc.

Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/01.RES.0000205761.22353.5f



ACE2-regulated pathways. Both ACE and ACE2 are involved in the production of the biologically active peptides Ang II and Ang1-7 from Ang I. Elevated levels of Ang II are known to be detrimental to the function of heart and kidney. The function of Ang1-9 is not well understood. The emerging picture of Ang1-7 function is of a key peptide involved in cardioprotection and renoprotection. From genetic experiments, it appears that ACE and ACE2 have complementary functions by negatively regulating different RAS products. The fine details of their regulatory function may differ depending on the local RAS environment.

angiotensin-converting enzyme (ACE) and ACE2, began only recently to unravel. ACE functions primarily as a "peptidyl dipeptidase," removing dipeptides from the C terminus of peptide substrates.<sup>8</sup> Its primary substrate was identified as Ang I. ACE processes the decapeptide Ang I to the 8-amino acid (aa) peptide Ang II (Figure). In contrast, ACE2 cleaves only a single amino acid from the C terminus of any given substrate. The role of ACE in regulation of cardiovascular function, fluid and electrolyte homeostasis is well established. Several small molecule inhibitors of human ACE are used for antihypertensive therapies,<sup>9</sup> lowering the risk of coronary heart disease and stroke, and treatments of cardiac failure and diabetic nephropathy.<sup>10</sup> Much less is known about the physiological function of ACE2.

ACE2 was initially found to be expressed in endothelia of the heart and in tubular epithelial cells of the kidney.<sup>11,12</sup> Subsequent studies using quantitative polymerase chain reaction have shown that ACE2 gene expression also occurs in the gastrointestinal tract<sup>13</sup> and, to a lesser extent, in other organs such as lungs.<sup>14</sup> Experiments showing that old ACE2-deficient mice develop progressively impaired heart functions that can be rescued by the loss of ACE have provided evidence for the direct involvement of the RAS in the modulation of cardiac contractility.<sup>15,16</sup> Additionally, the observation of ventricular tachycardia and heart block in ACE2 transgenic mice suggested a role of RAS in ventricular remodeling, supporting the clinical observations that ACE inhibitors have beneficial effects on cardiac remodeling and heart failure.<sup>17</sup> In addition to the heart, the RAS plays an important role in the control of kidney function.<sup>18</sup> For instance, ACE inhibitors and Ang II receptor antagonists can confer renoprotection in experimental and human diabetic nephropathy.<sup>19,20</sup> High expression levels of ACE2 in the normal kidney,<sup>12,13,21</sup> together with the observations of reduced levels of ACE2 in diabetic rats<sup>22</sup> and in human kidney diseases,<sup>23</sup> imply ACE2 involvement in kidney physiology and pathophysiology. In line with these observations, ACE2

mutant mice exhibit late-onset glomerulosclerosis and renal protein leakage.<sup>24</sup> Moreover, because it has been shown that ACE2 acts not only on Ang I and Ang II peptides, but also efficiently cleaves the C-terminal residues from several unrelated peptides such as apelin-13 or dynorphinA,<sup>12,25</sup> ACE2 functions may not be limited only to the RAS.

### Balancing the RAS Pathway

In the classic pathway of RAS, Ang II is a product of a "peptidyl dipeptidase" ACE. In this process, the decapeptide Ang I is converted by ACE to Ang II (Figure). Ang I is generated from the circulating precursor angiotensinogen (AGT) by the action of renin, an enzyme secreted from by juxtaglomerular cells at the renal afferent arterioles.<sup>26</sup> Ang II plays a central role as a potent regulator of fluid volumes, blood pressure regulation, and cardiovascular remodeling by binding to the Ang II G-protein-coupled receptors type 1 (AT<sub>1</sub>) and type 2 (AT<sub>2</sub>).<sup>19</sup> The majority of the cardiac and renal actions of Ang II are mediated by the AT<sub>1</sub> receptor, including vascular smooth muscle contraction, aldosterone secretion, diuretic responses, adrenergic stimulation, renal sodium reabsorption, and pressor and chronotropic responses.<sup>19</sup> Ang II also binds to AT<sub>2</sub> receptors, inducing a counter-regulatory vasodilation that is largely mediated by bradykinin and NO.<sup>20</sup> The emerging picture of ACE2 function is of a key enzyme catalyzing the cleavage of both Ang I and Ang II. ACE2 cleaves the C-terminal amino acid of Ang I to the nonapeptide angiotensin1-9 (Ang1-9).<sup>12</sup> Ang1-9 is thought to potentiate Ang II-mediated vasoconstriction in isolated rat aortic rings and to have vasopressor effects in conscious rats.<sup>27</sup> In rat and human plasma, Ang1-9 levels are twice those of Ang II.<sup>25,28</sup> Ang1-9 accumulates in animals treated with ACE inhibitors.<sup>29</sup> Also, Ang1-9 was found to augment bradykinin action on its B2 receptor by probably inducing conformational changes in the ACE/B2 receptor complex via interaction with ACE.<sup>30</sup> The biological function of Ang1-9 in heart and kidney is still not well defined. ACE2 also directly converts Ang II to Ang1-7.<sup>12,31-33</sup> In animals, Ang1-7 has been proposed to be an important regulator of cardiovascular and renal function promoting vasodilation, apoptosis, and growth arrest.<sup>34,35</sup> It is important to note that ACE and ACE2 are not the only enzymes involved in the RAS pathway; for example, chymases convert Ang I to Ang II, and other angiotensinases are known to hydrolyze Ang I to Ang1-7 or Ang1-9. Still, the unique patterns of Ang I metabolism by ACE and ACE2 may represent the biochemical and physiological counter-regulatory arms of the RAS in the regulation of cardiovascular and renal function. ACE2 seems to regulate Ang II production by ACE either by stimulating an alternative pathway for Ang I degradation or by facilitating the degradation of Ang II into Ang1-7. However, according to the feed-forward node enzymatic pathway, ACE determines both the production of Ang II and the degradation of Ang1-7, whereas ACE2, by facilitating the conversion of Ang II into Ang1-7, can regulate the net level of Ang II present in the tissue.<sup>36</sup> The peptide Ang1-7, through its recently identified receptor the mas oncogene product (MAS),<sup>37</sup> may stimulate NO synthase and counteract the potentially detrimental actions of Ang II via the AT<sub>1</sub> recep-

## ACE2 Substrates and Products

ACE2 Substrates/Products	Receptor	Cardiac and Renal Functions
Ang I	Unknown	Unknown
Asp Arg Val Tyr Ile His Pro Phe His Leu		
Ang1-9	Unknown	Vasoconstriction?
Asp Arg Val Tyr Ile His Pro Phe His		
Ang II Asp Arg Val Tyr Ile His Pro Phe	G-protein-coupled AT1 and AT2 receptors	Vasoconstrictor, cardiomyocyte hypertrophy, fibroblasts proliferation, cardiac and cardiomyocyte contractility, regulation of glomerular hemodynamics, and proteinuria
Ang1-7 Asp Arg Val Tyr Ile His Pro	G-protein-coupled mas receptor, other receptors?	Vasodilator, inhibition of cell growth, sodium and water flux, reduction in glomerular filtration
Apelin-36 c term-Ser His Lys Gly Pro Met Pro Phe Apelin-13 Gln Arg Pro Arg Leu Ser His Lys Gly Pro Met Pro Phe	APJ receptor	Vasoconstriction, vasodilation, water intake, myocardial contractility, regulation of stroke volume and cardiac output
des-Arg <sup>8</sup> -bradykinin	G-protein-coupled receptors B1	Induced during inflammation and ischemia
Arg Pro Pro Gly Phe Ser Pro Phe	G-protein-coupled receptors	Protective role in the development of hypertension and renal and cardiovascular complications
Lys des-Arg <sup>8</sup> -bradykinin Lys Arg Pro Pro Gly Phe Ser Pro Phe	B1/G-protein-coupled receptors B2	
Dynorphin A Tyr Gly Gly Phe Leu Arg Arg Ile Arg Pro Lys Leu Lys $\beta$ casamorphin Tyr Pro Phe Val Glu Pro Ile	$\kappa$ and $\delta$ G-protein-coupled opioid receptors	Pain perception, cardiomyocyte contractility, arterial pressure
Neurotensin pGlu-Leu Tyr Glu Asn Lys Pro Arg	G-protein-coupled neurotensin receptors	Ventricular contractility, regulation of renin release, sodium excretion

ACE2 functions as a carboxypeptidase with a preference for C-terminal Leu or Phe. The ACE2 substrates, products, and their receptors, if known, are indicated. The cardiac and renal functions of ACE2 substrates or products are not always well defined.

tor.<sup>38</sup> The effects of Ang1-7 may also involve binding to AT<sub>1</sub> receptor and augmenting bradykinin binding to the bradykinin B<sub>2</sub> receptor.<sup>39</sup> A major pathway of Ang1-7 degradation, whereby the peptide is converted to inactive fragments, is via ACE itself. Therefore, ACE inhibition can increase Ang1-7 levels while simultaneously reducing Ang II. Thus, it appears that ACE2 is a negative regulator of the RAS and counterbalances ACE function.

## Additional ACE2 Substrates

In addition to its activity as an enzyme converting Ang II to Ang1-7 or Ang I to Ang1-9, ACE2 can remove in vitro assays the C-terminal residue from apelin and other vasoactive peptides such as neurotensin, kinetensin (a neurotensin-related peptide), and des-Arg bradykinin (Table). Indeed, ACE2 acts on apelin-13 and apelin-36 peptides with high catalytic efficiency.<sup>33</sup> These two forms of apelin were recently identified as endogenous ligands for the human APJ receptor, the homolog of the angiotensin receptor AT<sub>1</sub>.<sup>40</sup> However, APJ knockout mice showed a rather minor increase in their vasopressor response to Ang II, nevertheless, suggesting a counter-regulatory role in relation to the RAS.<sup>41</sup> Apelin also induces an increase in myocardial contractility and a reduction of vasomotor tone.<sup>42</sup> Although the increase in contractility seems to depend on an activation of Na<sup>+</sup>/H<sup>+</sup> and Na<sup>+</sup>/Ca<sup>2+</sup> exchangers, vasodilation is attributed to a release of NO from the vascular endothelial cells.<sup>43</sup> When apelin is given acutely, the decrease in preload favors the reduction of stroke volume and cardiac output in spite of an increased contractility. Chronic administration of apelin significantly increases cardiac output without the occurrence of cardiac

hypertrophy. However, potential chronic side effects of apelin administration need to be determined.<sup>43</sup>

Two opioid peptides, dynorphin A and  $\beta$ -casamorphin, are also substrates of ACE2.<sup>33</sup> (Table). These peptides activate  $\kappa$  and  $\delta$  opioid G-protein-coupled receptors that regulate pain perception and, among other functions, may have negative effects on cardiomyocyte contractility.<sup>44</sup> Opioid peptides and their receptors show broad distribution in the various brain areas but are also expressed at sites that control the cardiovascular system.<sup>45,46</sup> Potent cardiovascular effects have been reported after central administration of opioid peptides.<sup>47</sup> For instance, intracerebroventricular administration of  $\beta$ -endorphin decreases the lumbar sympathetic nerve activity and mean arterial pressure in anesthetized rats.<sup>48</sup> However, it should be noted that studies with various opioid agonists are conflicting.

The kinin metabolites, the nonapeptide bradykinin, and its biologically active metabolite exert their effects by selective activation of the two kinin receptor types: B<sub>1</sub> and B<sub>2</sub>. The bradykinin B<sub>2</sub> receptor is constitutively expressed in most human tissues and mediates the majority of the visceral and vascular actions of bradykinin, whereas the bradykinin B<sub>1</sub> receptor is expressed mainly under pathological conditions such as inflammation and sepsis, being selectively activated by des-Arg<sup>9</sup> metabolites of the kinins.<sup>49,50</sup> ACE2 does not metabolize bradykinin but inactivates both des-Arg<sup>9</sup>-bradykinin and lys-des-Arg<sup>9</sup>-bradykinin.<sup>12,51</sup> In various animal models and in humans, it has been shown that the stimulation of bradykinin B<sub>2</sub> receptors is not only implicated in the pathogenesis of inflammation, pain and tissue injury, but also triggers cardioprotective and renoprotective func-

tions.<sup>52,53</sup> In conclusion, although the biological peptides Ang I and Ang II are principal ACE2 substrates, ACE2 can cleave multiple other target peptides such as apelin-13, dynorphin A, or des-Arg<sup>8</sup>-bradykinin. Thus, although ACE2 functions have been primarily attributed to the regulation of the RAS, Ang II and Ang I–7 are probably only part of the ACE2 story, and other ACE2 substrates may contribute to the *in vivo* functions of ACE2.

### Cardiac Functions

For a number of years, ACE and its main biologically active peptide Ang II have assumed a central position in the cardiac RAS. With the discovery of ACE2, a new regulator entered the established metabolic RAS pathways. Components of the local cardiac RAS are heterologously distributed on different cell types within the heart.<sup>54</sup> For instance, AGT is primarily distributed in atrial muscle and the neuronal fibers of the conduction system, with small amounts in the subendocardial region of the ventricle.<sup>18</sup> In contrast, ACE is primarily expressed by coronary endothelial cells and cardiac fibroblasts.<sup>18</sup> Additionally, ACE expression can be detected in all four heart valves, coronary blood vessels, the aorta pulmonary artery, endocardium, as well as epicardium.<sup>55,56</sup> ACE2 is localized to the endothelium and smooth muscle cells of most intramyocardial vessels, including capillaries, venules, and medium-sized coronary arteries and arterioles.<sup>57</sup> Furthermore, ACE2 protein expression was detected in cardiac myocytes from failing human hearts.<sup>57</sup> It is important to note that although all the components of RAS are present in the heart, not all of them are believed to be synthesized in heart. For example, the question whether renin is synthesized in heart or is derived primarily from circulation remains still unresolved.<sup>58</sup> Together, the final balance of biologically active peptides produced within local heart environment may depend on the coexpression and the relative levels of ACE and ACE2 within different cell types.

### Cardiac Contractility

Although hearts from young *ace2* mutant mice are functionally normal, hearts of old *ace2*-deficient mice in this particular mouse background display a reduction in cardiac contractility as demonstrated by 40% reduction in fractional shortening and velocity of circumferential shortening (heart rate corrected) with slight ventricular dilation.<sup>15</sup> The significance of ACE2 in regulating cardiac function is further highlighted by the thinning of the left ventricular wall in aged *ace2* mutant mice. This progressive cardiac dysfunction occurred without myocardial fibrosis or hypertrophy and in the absence of the myosin heavy chain isoform switches typically found in other animal models of heart failure. Thus, one may speculate that the observed phenotype closely resembles the defective heart found in patients with cardiac stunning/hibernation.<sup>59</sup> Cardiac stunning and hibernation reflect adaptive responses to prolonged tissue hypoxia that occurs in coronary artery disease or after bypass surgery.<sup>60</sup> In these human diseases and related animal models, chronic hypoxic conditions lead to compensatory changes in myocyte metabolism,<sup>61</sup> upregulation of hypoxia-induced genes,<sup>62</sup> and reduced heart function.<sup>63</sup> Accordingly, the hearts of *ace2* null

mice show upregulation of mRNA expression of hypoxia-inducible genes such as *BNIP3*<sup>62</sup> and *PAI-1*.<sup>63</sup> The magnitude of increased expression of these hypoxia-inducible genes resembles previously observed levels in other hypoxic models such as the myocyte-specific vascular endothelial growth factor mutant mice.<sup>64</sup> However, the link between cardiac stunning/hibernation and the heart defect observed in *ace2* knockout mice has to be investigated further. Whether ACE2 expression levels indeed change under conditions of hypoxia remains to be demonstrated.

ACE2 knockout mice show also increased local heart Ang II levels.<sup>15</sup> Interestingly, both the cardiac phenotype and increased Ang II levels were completely reversed by additional deletion of *ace* gene (ie, ablation of ACE expression on an *ace2* mutant background abolished the cardiac dysfunction phenotype of *ace2* single knockout mice).<sup>15</sup> The heart function of *ace/ace2* double mutant mice was similar to that in *ace* single mutant and wild-type littermates. The normal cardiac functions of *ace/ace2* double mutant mice suggest that the catalytic products of ACE account for the observed contractile impairment of old *ace2* single mutant mice. These observations for the first time demonstrated at the genetic level that ACE2 counterbalances the enzymatic actions of ACE. It seems that increased local cardiac Ang II might have been the cause for the cardiac abnormalities in *ace2*-deficient mice. However, it remains unclear why despite the elevated plasma and heart Ang II levels, the heart of the *ace2*-deficient mice did not show any evidence for cardiac hypertrophy. In fact, it is well established that cardiac myocytes express Ang II receptors and undergo hypertrophy in response to Ang II. However, *in vivo*, elevated cardiac Ang II levels alone do not directly induce cardiac hypertrophy but do increase interstitial fibrosis.<sup>65</sup> Thus, it is important to note that Ang II-independent pathways could also play an important role in ACE/ACE2-regulated heart function.

### ACE2 and Heart Conduction

In several published studies, Ang II has also been implicated in conduction abnormalities, although some results appear contradictory. Slowed conduction was associated with increased myocardial and plasma ACE activity. Moreover, administration of an ACE inhibitor improved conduction velocities in cardiomyopathy using a Syrian hamster model.<sup>66–68</sup> These observations suggest that Ang II slows cardiac conduction. This conclusion is further supported by the finding of slowed ventricular conduction in mice overexpressing the AT<sub>1</sub> receptor.<sup>69</sup> However, in contrast, in cardiac myocyte cultures, Ang II stimulated an increase in connexin43, a protein implicated in the upregulation of cardiac conduction,<sup>70</sup> implying that Ang II may accelerate cardiac conduction. Interestingly, in *ace2* null mice, elevated levels of Ang II did not affect normal conductivity, and the mice appear to have a normal life span, at least under nonstress laboratory conditions. However, overexpression of ACE2, under the control of the myosin promoter, caused conduction disturbances that in some animals degenerated into ventricular fibrillation with arrest and sudden death.<sup>17</sup> The severity of this phenotype correlated with the ACE2 expression levels; mice with higher expression of ACE2 were dying by 5 weeks

of age, whereas moderate expression of ACE2 extended their survival to 23 weeks. The question whether cardiac conduction is in fact influenced by the RAS under physiological condition has to be re-examined because it has been proposed that Ang1-7, a main product of ACE2 enzymatic activity in the heart, has antiarrhythmic actions.<sup>71</sup> However, it is important to note that transgenic overexpression of ACE2 without ACE upregulation may shift the balance from the production of the cardioprotective and antiarrhythmic Ang1-7 to Ang1-9. Whether ACE2 plays indeed a role in cardiac conduction system should be assessed in mutant animals under conditions of stress or chronic injury.

### ACE2 and the Failing Heart

Accumulating evidence indicates that the local cardiac RAS and myocardial Ang II production is activated in myocardial infarction.<sup>72-74</sup> Indeed, increased cardiac expression of AGT, ACE, and AT<sub>1</sub> receptor proteins, increased ACE activity, as well as elevated Ang II levels have been reported in infarcted hearts.<sup>72</sup> Moreover, ACE2 expression increases in the infarct zone, followed by increased ACE2 expression in the myocardium surrounding the ischemic zone after coronary artery ligation in rats.<sup>57</sup> Blockade of AT<sub>1</sub> receptors by losartan or olmesartan for 28 days after occlusion of a coronary artery resulted in a significant increase in cardiac ACE2 mRNA expression as well as increased ACE2 activity.<sup>36</sup> Furthermore, inhibition of Ang II synthesis by 12-day oral administration of lisinopril increased cardiac ACE2 gene transcription.<sup>66</sup> Moreover, ACE2 gene expression and activity are also significantly increased in the failing human heart.<sup>75,76</sup> The identification of ACE2 in the failing heart highlights its possible role in opposing the effects of Ang II.

The hypothesis that ACE2 and its product Ang1-7 may oppose the actions of Ang II was further supported by studies using normotensive Lewis rats.<sup>77</sup> After coronary artery ligation, cardiac hypertrophy and left ventricular dysfunction were accompanied by increased plasma concentrations of Ang I, Ang II, and Ang1-7, and downregulation of cardiac AT<sub>1</sub> receptor expression. Treatment with the AT<sub>1</sub> receptor antagonists losartan and olmesartan reversed cardiac hypertrophy and improved ventricular contractility. Both AT<sub>1</sub> receptor blockers further increased angiotensin peptide concentrations, returned AT<sub>1</sub> receptor expression to normal, and increased ACE2 expression in the heart.<sup>77</sup> It is important to note that in both studies in Lewis rats, cardiac ACE and ACE2 expression were unchanged in response to coronary artery ligation in the absence of drug treatment. Whether ACE2 expression has affected the severity or outcome of myocardial infarction remains contentious. However, what has emerged from recent studies appears to be the involvement of ACE2 in increasing the content of cardiac Ang1-7. Because Ang1-7 is formed within the heart after AT<sub>1</sub> receptor blockade, ACE2 may be responsible for the beneficial actions observed on such a treatment on cardiac function. Furthermore, although ACE inhibitors were originally developed to suppress the formation of Ang II, recent studies suggest that part of their beneficial effect in cardiovascular diseases may be attributed to the elevation of plasma Ang1-7 levels.<sup>78-80</sup> Whether Ang1-7 indeed contributes to heart disease or is

simply a byproduct of the local RAS activation needs to be examined further (eg, in mice lacking the Ang1-7 receptor).

### Renal Function of ACE2

A paradigm shift has occurred in recent years from an emphasis on the role of the systemic circulating RAS in the regulation of fluid and electrolyte balance and arterial pressure to focus on the local tissue RAS in kidneys. In the kidney, number of components of the RAS such as renin, AGT, and ACE mRNA are colocalized in a site-specific manner.<sup>81-84</sup> Furthermore, the hypothesis that Ang II plays a tissue-specific role in the kidney is consistent with the finding that Ang II receptors are localized to renal arterioles, glomerular mesangial cells, and on the basolateral and apical membranes of proximal tubule cells.<sup>21,85</sup>

Within the kidney, ACE2 has a distribution similar to ACE. ACE2 is present in distal tubules, proximal tubules, and to a much lesser extent in glomeruli, as assessed by both gene and protein expression.<sup>21,86-88</sup> Interestingly, most of the intrarenal AGT is localized in the proximal tubule,<sup>82-84,89-91</sup> and AGT is secreted directly into the tubule lumen, where it serves as a substrate for renin or renin-like enzymes.<sup>89,91</sup> Because ACE is located on the proximal tubule cell brush border, it can promptly convert Ang I to Ang II.<sup>92,93</sup> Renal interstitial fluid contains a 1000-fold higher level of Ang II than plasma. However, as shown recently, ACE seems not to be the only enzyme contributing to Ang II formation in kidney, suggesting that besides other "angiotensinases," the intrarenal levels of Ang II may be also regulated by ACE2. For instance, incubation of isolated proximal tubules with Ang I led to generation of Ang II as well as Ang1-7 and Ang1-9. Generation of Ang1-7 was blocked by the ACE2 inhibitor DX600. Although *in vitro* studies indicate that ACE2 has 400-fold greater efficacy to convert Ang II to Ang1-7 compared with the conversion of Ang I to Ang1-9<sup>31,33</sup> or the conversion of other peptide substrates, incubation of proximal tubules with Ang II or luminal perfusion of Ang II did not result in detection of Ang1-7.<sup>88</sup> Nonetheless, ACE2-regulated Ang1-7 production *in vivo* may represent an important component of the proximal tubular RAS. Several studies have documented that Ang1-7 is a major biologically active peptide in kidneys.<sup>80,94-96</sup> However, the role of Ang1-7 remains somewhat controversial. In most situations, Ang1-7 opposes the actions of Ang II. For instance, Ang1-7 infusion produced a marked natriuresis in the kidney of normotensive rats and dogs.<sup>34,96</sup> Moreover, it has been reported that Ang1-7 causes afferent arteriolar vasodilatation,<sup>97</sup> and even if devoid of any vasodilator actions by itself, it antagonizes the renal vasoconstrictor effects of Ang II. Furthermore, treatment with either an Ang1-7 monoclonal antibody or with the selective Ang1-7 receptor antagonist 7-D-Ala-Ang1-7 elicited a dose-dependent rise in blood pressure and reversed to a significant degree the blood pressure-lowering effects of ACE inhibitors in hypertensive rats.<sup>34,98</sup> In contrast to these experiments, it has been shown that Ang1-7 exhibits antidiuretic actions in water-loaded rats<sup>39</sup> and stimulates renal tubular sodium reabsorption in normotensive rats.<sup>99</sup> Moreover, it has been reported that Ang1-7 does not exert vasodilator or Ang II, opposing actions in the renal circulation.<sup>97</sup> That ACE2 may

be functionally linked to the tissue production of Ang1-7 is supported by the increased coexpression as well as colocalization of ACE2 protein and Ang1-7 in the renal proximal tubules of spontaneously hypertensive rats on treatment with the vasopeptidase inhibitor omapatrilat.<sup>100</sup> Omapatrilat targets both ACE and neprilysin but not ACE2. Furthermore, mRNA ACE2 levels in the kidney increased 75% after Omapatrilat treatment. Similar findings were reported in pregnant rats.<sup>101</sup> Pregnancy increases the levels of both Ang1-7 and ACE2 in the renal tubules without affecting the overall pattern of ACE2 distribution. Increased levels of Ang1-7 in association with increased ACE2 expression support the notion that ACE2 may indeed play an important role in local kidney RAS.<sup>18</sup> Together, these findings suggest that Ang1-7 might be an important component of the RAS and a critical link in mediating the negative regulatory feedback between ACE and ACE2. To what extent ACE2 may contribute to these divergent functions of Ang1-7 in the kidney remains unclear.

Few data are available on the functional role of ACE2 in the kidney. The first reported data on ACE2 in kidneys showed that hypertension correlates with ACE2 expression.<sup>15</sup> For example, *ace2* mRNA levels in the kidneys of salt-sensitive Sabra hypertensive (SBH/y) rats were lower than in the normotensive salt-resistant Sabra normotensive (SBN/y) rats. In addition, ACE2 protein expression was also markedly reduced in SBH/y animals that were fed a normal diet. Increase in blood pressure of SBH/y rats after a 4-week diet of DOCA salt correlated with a further decrease in ACE2 protein expression. ACE2 protein levels were also significantly decreased in the kidneys of spontaneously hypertensive stroke-prone and spontaneously hypertensive rats compared with their Wistar Kyoto controls.<sup>15</sup> Recently, it has been reported that ACE2 levels are reduced in experimental diabetic nephropathy.<sup>21</sup> It is not yet known whether this reduction in ACE2 is of pathophysiological significance in diabetic nephropathy, but one could postulate that ACE2 deficiency leads to a local increase in tubular Ang II, with subsequent effects such as promotion of interstitial fibrosis. For instance, local increases in Ang II have been also reported in damaged tubules in various experimental models of progressive renal disease<sup>102</sup> such as in renal ablation,<sup>103</sup> passive Heymann nephritis,<sup>104</sup> anti-Thy1 glomerulonephritis,<sup>105</sup> anti-GBM nephritis,<sup>106,107</sup> and also glomerulosclerosis.<sup>108</sup> For instance, in glomerulosclerosis, it has been suggested that elevated Ang II levels might contribute to late development of glomerular injury and proteinuria.<sup>34,108</sup> These studies support the view that local unopposed action of the ACE enzyme is generally associated with enhanced Ang II formation, resulting in increased renal damage. In line with this hypothesis, ACE inhibitors and AT<sub>1</sub> receptor antagonist are known to reduce such renal injury and are used in the clinic for diabetic nephropathy. In humans, increased expression of ACE2 in glomerular and peritubular endothelium has been consistently observed in diseased kidneys across different diagnosis categories as well as renal transplants.<sup>23,109</sup> Furthermore, mice at an early stage of diabetes exhibited increased ACE2 protein in renal cortical tubules coupled with profound reduction in renal expression of ACE.<sup>66</sup> These data are

consistent with the assumption that increased expression of ACE2 may reflect a protective mechanism. Because Ang II is thought to play an important role in the progression of diabetic nephropathy, decreased renal ACE activity tied with increased renal ACE2 expression may be protective for the kidneys in the early phases of diabetes by limiting the renal accumulation of Ang II and favoring Ang1-7 formation. Interestingly, the decrease in ACE activity associated with an increase in ACE2 protein expression resembles the pattern seen after administration of a renoprotective drug, ramipril, to diabetic rats.<sup>21</sup> However, increased ACE2 protein expression in renal cortical tubules from the young diabetic mice does not exclude the possibility of an ACE2 reduction later during the development of nephropathy. In fact, decreased ACE2 expression in concert with increased ACE activity may foster kidney damage in diabetes.<sup>21</sup> Importantly, it has been shown recently that old *ace2* mutant mice, in particular males, develop Ang II-dependent glomerulosclerosis that resembles diabetic nephropathy in humans.<sup>24</sup>

### Concluding Remarks

The transmembrane protease ACE2 has emerged as a negative regulator of the RAS that counterbalances the multiple functions of ACE. Genetic data have shown that ACE2 plays a protective role in heart and kidney functions. In addition to the critical and multiple functions of Ang II, it is becoming clear that Ang1-7 and possibly Ang1-9 are additional major biologically active products of the RAS. ACE2 does not only function in the metabolism of RAS peptides but also in the catalysis of opioid peptides, apelin, neurotensin, or kinetensin. Thus, enhancing ACE2 function might have effects and benefits that extend beyond the known functions of Ang II and its receptor. Understanding the physiological roles of ACE2 in myocardial function and its contribution to kidney damage may ultimately lead to the development of new therapeutic agents.

### Acknowledgments

This work was supported by grants from the National Bank of Austria, the Austrian Ministry of Science and Education, IMBA, an EU Marie Curie Excellence grant, and EUGeneHeart to J.M.P. We thank M.J. Crackower, R. Sarao, Yumiko Imai, Keiji Kuba, and many others for their contributions.

### References

1. Peach MJ. Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol Rev*. 1977;57:313-370.
2. Gavras H, Lever AF, Brown JJ, Macadam RF, Robertson JJ. Acute renal failure, tubular necrosis, and myocardial infarction induced in the rabbit by intravenous angiotensin II. *Lancet*. 1971;2.
3. Vaughan D. Angiotensin, fibrinolysis, and vascular homeostasis. *Am J Cardiol*. 2001;87:18C-24C.
4. Wolf G. Free radical production and angiotensin. *Curr Hypertens Rep*. 2002;2:167-173.
5. Ding G, Reddy K, Kapasi AA, Franki N, Gibbons N, Kasinath BS, Singhal PC. Angiotensin II induces apoptosis in rat glomerular epithelial cells. *Am J Physiol Renal Physiol*. 2002;283:F173-F180.
6. Gavras H, Brunner HR. Role of angiotensin and its inhibition in hypertension, ischemic heart disease, and heart failure. *Hypertension*. 2001;37:342-345.
7. Gavras I, Gavras H. Angiotensin II as a cardiovascular risk factor. *J Hum Hypertens*. 2002;16:S2-S6.
8. Skeggs LT. The preparation and function of the hypertension converting enzyme. *J Exp Med*. 1956;103:295-299.

9. Cushman DW, Ondetto MA. Inhibitors of angiotensin-converting enzyme for treatment of hypertension. *Biochem Pharmacol*. 1980;29:1871-1877.
10. Turner AJ, Hooper NM. The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol Sci*. 2002;23:177-183.
11. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, and Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem*. 2000;275:33238-33243.
12. Donoghue M, Hsieh F, Barones E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Joyasacian R, Breitbart RE, Acton, SA novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*. 2002;87:E1-E9.
13. Harner D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett*. 2002;532:107-110.
14. Komatsu T, Suzuki Y, Imai J, Sugano S, Hida M, Tanigami A, Muroi S, Yamada Y, Hanaka KK. Molecular cloning, mRNA expression and chromosomal localization of mouse angiotensin-converting enzyme-related carboxypeptidase (mACE2). *FEBS Lett*. 2002;532:127-130.
15. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-Dos-Santos AJ, Da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backs PH, Yagil Y, Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature*. 2002;415:822-828.
16. Danilczyk U, Eriksson U, Crackower MA, Penninger JM. A story of two ACEs. *J Mol Med*. 2003;81:227-234.
17. Donoghue M, Wakimoto M, Maguire CT, Acton S, Hales P, Stagliano N, Fairchild-Huntress V, Xu J, Lorenz JN, Kadambi V, Berl CI, Breitbart RE. Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins. *J Mol Cell Cardiol*. 2003;35:1043-1053.
18. Carey RM, Siragy HM. Newly recognized components of the renin-angiotensin system: potential role in cardiovascular and renal regulation. *Endocr Rev*. 2005;26:261-271.
19. de Gasparo M, Catt KJ, Wright JW, Unger T. The angiotensin II receptors. *Pharmacol Rev*. 2000;52:415-472.
20. Joergens O, Dendorfer A, Dominik P. Cardiovascular and renal function of angiotensin II type-2 receptor. *Cardiovascular Res*. 2004;62:460-467.
21. Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME. Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension*. 2003;41:392-397.
22. Douglas JG. Angiotensin receptor subtypes in the kidney cortex. *Am J Physiol*. 1987;253:F1-F7.
23. Lely AT, Hamming I, van Goor H, Navis GJ. Renal ACE2 expression in human kidney disease. *J Pathol*. 2004;204:587-593.
24. Oudit GY, Herzberg AM, Kassir Z, Wong D, Reich H, Khokha R, Crackower MA, Backs PH, Penninger JM, Scholey W. Loss of ACE2 leads to the development of angiotensin II-dependent glomerulosclerosis. *Am J Physiol*. In press.
25. Johnson H, Kouris S, Waters J, Drummer OH. Radioimmunoassay for immunoreactive [Ile-Leu]10-angiotensin I. *Peptides (Elmsford)*. 1989;10:489-492.
26. Inagami T. The renin angiotensin system. *Essays Biochem*. 1994;28:147-164.
27. Huang L, Sexton DL, Skogerson K, Devlin M, Smith R, Sanyal I, Parry T, Kent R, Enright J, Wu Q-L, Conley G, DeOliveira D, Morganello L, Ducar M, Wescott CR, Ladner RC. Novel peptide inhibitors of angiotensin-converting enzyme 2. *J Biol Chem*. 2003;278:15532-15540.
28. Oparil S, Tregear GW, Koerner T, Barnes BA, Haber E. Mechanism of pulmonary conversion of angiotensin I to angiotensin II in the dog. *Circ Res*. 1971;29:682-690.
29. Drummer OH, Kouris S, Johnson H. Effect of chronic enalapril treatment on enzymes responsible for the catabolism of angiotensin I and formation of angiotensin II. *Biochem Pharmacol*. 1990;39:513-518.
30. Erdos EG, Jackman HL, Brovokovich V, Tan F, Deddish PA. Products of angiotensin I hydrolysis by human cardiac enzymes potentiate bradykinin. *J Mol Cell Cardiol*. 2002;34:1569-1576.
31. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J*. 2004;383:45-51.
32. Lin Q, Keller RS, Weaver B, Zisman LS. Interaction of ACE2 and integrin  $\beta_1$  in failing human heart. *Biochim Biophys Acta*. 2004;1689:175-178.
33. Vickers C, Hales P, Kaushik V, Dick L, Gavis J, Godbout JTK, Parsons T, Barones E, Hsieh F, Acton S, Patane M, Nichols A, Tuminello P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*. 2002;277:14838-14843.
34. Iyer SN, Averill DB, Chappell MC, Yamada K, Allred AJ, Ferrario CM. Contribution of angiotensin-(1-7) to blood pressure regulation in salt-depleted hypertensive rats. *Hypertension*. 2000;36:417-422.
35. Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension*. 2002;39:799-802.
36. Ferrario CM, Trask AJ, Jessup JA. Advances in the biochemical and functional roles of angiotensin converting enzyme 2 and angiotensin-(1-7) in the regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol*. 2005;289:H2281-H2290.
37. Santos RA, Simoes E, Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnolo-Santos MJ, Selverhausen HP, Spehr R, Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258-8263.
38. Ferrario CM. There is more to discover about angiotensin converting enzyme. *Hypertension*. 2003;41:390-391.
39. Santos RA, Campagnolo-Santos MJ, Baracho NC, Fontes MA, Silva LC, Neves LA, Oliveira DR, Caligaris SM, Rodrigues AR, Groten C Jr, et al. Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors. *Brain Res Bull*. 1994;35:293-298.
40. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habeta Y, Hinuma S, Kitada C, Honda S, Kurokawa T, Onda H, Nishimura O, Fujino M. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem*. 2000;275:21061-21067.
41. Ishida J, Hashimoto T, Hashimoto Y, Nishiwaki S, Iguchi T, Harada S, Sugaya T, Matsuzaki H, Yamamoto R, Shioh N, Okumishi H, Kihara M, Umehura S, Sugiyama F, Yagami K, Kasuya Y, Mochizuki N, Fukunaga A. Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. *J Biol Chem*. 2004;279:18279.
42. Tatemoto K, Takayama K, Zou MX, Kumaki J, Zhang W, Kumano K, Fujimura M. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept*. 2001;99:87-92.
43. Ashley EA, Powers J, Chen M, Kundu R, Finsterbach T, Caffarelli A, Deng A, Eichhorn J, Mahajan R, Agrawal R, Greve J, Robbins R, Patterson AJ, Bernstein D, Quertemont T. The endogenous peptide apelin potentially improves cardiac contractility and reduces cardiac loading in vivo. *Cardiovasc Res*. 2005;65:73-82.
44. Ventura C, Spurgeon H, Lakatta EG, Guarnieri C, Capogrossi MC. Kappa and delta opioid receptor stimulation affects cardiac myocyte function and Ca<sup>2+</sup> release from an intracellular pool in myocytes and neurons. *Circ Res*. 1992;70:66-81.
45. Aweh SF, Kuhar MJ. Autoradiographic localization of opiate receptors in rat brain. *Brain Res*. 1977;129:1-12.
46. Fallon JR, Leslie FM. Distribution of dynorphin and enkephalin peptides in the rat brain. *J Comp Neurol*. 1986;249:293-336.
47. Siren AL, Feuerstein G. Hypothalamic opioid mu-receptors regulate discrete hemodynamic functions in the conscious rat. *Neuropharmacology*. 1991;30:143-152.
48. Dunbar JC, Lu H. Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioid interactions. *Peptides (Elmsford)*. 2002;21:211-217.
49. Regoli D, Barabe J. Pharmacology of BK and related kinins. *Pharmacol Rev*. 1980;32:1-46.
50. McIntosh PG, Perreni M, Abulawia A, Kinin B1 receptors and the cardiovascular system: regulation of expression and function. *Cardiovasc Res*. 2000;48:194-210.
51. Oudit GY, Crackower MA, Backs PH, Penninger JM. The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc Med*. 2003;13:93-101.
52. Heitsch H. The therapeutic potential of bradykinin B2 receptor agonists in the treatment of cardiovascular disease. *Expert Opin Invest Drugs*. 2003;12:759-770.
53. Wang D, Yoshida H, Song Q, Chao L, Chao J. Enhanced renal function in bradykinin (B2) receptor transgenic mice. *Am J Physiol Renal Physiol*. 2000;278:F484-F491.

54. Sawa H, Tokuchi F, Mochizuki N, Endo Y, Furuta Y, Shinohara T, Takada A, Kawaguchi H, Yasuda H, Nakashima K. Expression of the angiotensinogen gene and localization of its protein in the human heart. *Circulation*. 1992;86:138–146.
55. Yamada HF, Fabris B, Allen AM, Jackson B, Johnston CI, Mendelsohn AO. Localization of angiotensin converting enzyme in rat heart. *Circ Res*. 1991;68:141–149.
56. Pagliaro P, Penn C. Rethinking the renin-Angiotensin system and its role in cardiovascular regulation. *Cardiovasc Drugs Ther*. 2005;19:77–87.
57. Burrell LM, Risanis J, Kubota E, Dean RG, MacDonald PS, Lu S, Tikellis CH, Grant SL, Lew RA, Smith AJ, Cooper ME, Johnston CI. Myocardial infarction increases ACE2 expression in rat and humans. *Eur Heart J*. 2005;26:369–375.
58. Dauter AH, Saris JJ, Schuit MP, van Kats JP. Is there a local renin-angiotensin system in the heart? *Cardiovasc Res*. 1999;44:252–265.
59. Eriksson U, Danilczyk U, Penninger JM. Just the beginning: novel functions for angiotensin-converting enzymes. *Curr Biol*. 2002;12:R745–R752.
60. Heusch G. Hibernating myocardium. *Physiol Rev*. 1998;78:1055–1085.
61. Murphy AM, Kogler H, Georgakopoulos D, McDonough JL, Kass DA, Van Eyk JE, Marban E. Transgenic mouse model of stunned myocardium. *Science*. 2000;287:488–491.
62. Sower HM, Rutcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNP1 and NIX in human tumors. *Cancer Res*. 2001;61:6669–6673.
63. Kietzmann T, Roth U, Jungermann K. Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element: heterologous hypoxia-inducible factor-1 in rat hepatocytes. *Blood*. 1999;94:4177–4185.
64. Giordano FJ, Gerber HP, Williams SP, vanBruggen N, Bunting S, Ruiz-Lozano P, Gu Y, Nath AK, Huang Y, Hickey R, Dalton N, Peterson KL, Ross J Jr, Chien KR, Ferrara N. A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. *Proc Natl Acad Sci U S A*. 2001;98:5780–5785.
65. van Kats JP, Mehot D, Paradis P, Silversides DW, Reudelhuber TL. Use of a biological peptide pump to study chronic peptide hormone action in transgenic mice. Direct and indirect effects of angiotensin II on the heart. *J Biol Chem*. 2001;276:44012–44017.
66. De Mello WC, Cherny RC, Manivannan S. Electrophysiologic and morphologic abnormalities in the failing heart: effect of enalapril on the electrical properties. *J Card Fail*. 1997;3:53–61.
67. De Mello WC. Cell coupling and impulse propagation in the failing heart. *J Cardiovasc Electrophysiol*. 1999;10:1409–1420.
68. De Mello WC, Crespo MJ. Correlation between changes in morphology, electrical properties, and angiotensin-converting enzyme activity in the failing heart. *Eur J Pharmacol*. 1999;378:187–194.
69. Hein L, Stevens ME, Barsh GS, Pratt RE, Koblik BK, Dzau VJ. Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc Natl Acad Sci U S A*. 1997;94:6391–6396.
70. Dodge SM, Beardslee MA, Darow BJ, Green KG, Beyer EC, Saffitz JE. Effects of angiotensin II on expression of the gap junction channel protein connexin43 in neonatal rat ventricular myocytes. *J Am Coll Cardiol*. 1998;32.
71. Ferreira AJ, Santos RA, Almeida AP. Angiotensin-(1–7): cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension*. 2001;38:665–678.
72. Johnston CI. Tissue angiotensin converting enzyme in cardiac and vascular hypertrophy, repair, and remodeling. *Hypertension*. 1994;23:258–268.
73. Passier RC, Smits JF, Verloyten MJ, Daemen MJ. Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. *Am J Physiol*. 1996;271:H1040–H1048.
74. Silvestre JS, Heymes C, Oubenaissa A, Robert V, Aupetit-Faissant B, Carayon A, Swynghedauw B, Delacaye C. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation*. 1999;99:2694–2701.
75. Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR, Canver CC. Increased angiotensin-(1–7) forming activity in failing human heart ventricles. Evidence for upregulation of the angiotensin-converting enzyme homologue ACE2. *Circulation*. 2003;108:1707.
76. Goulter A, Goddard MJ, Allen JC, Clark KL. ACE2 gene expression is up-regulated in the human failing heart. *BMC Med*. 2004;2:19.
77. Ishiyama Y, Gallagher PE, Averill DB, Tallant EA, Brushinin KB, Ferrario CM. Up-regulation of angiotensin converting enzyme-2 after myocardial infarction by blockade of angiotensin II receptors. *Hypertension*. 2004;43:970–976.
78. Iyer SN, Chappell MC, Averill DB, Diz DI, Ferrario CM. Vasodilator actions of angiotensin-(1–7) unmasked during combined treatment with lisinopril and losartan. *Hypertension*. 1998;31:699–705.
79. Lawrence AC, Evin G, Kladis A, Campbell DJ. An alternative strategy for the radioimmunoassay of angiotensin peptides using amino-terminal-directed antisera: measurement of eight angiotensin peptides in human plasma. *J Hypertens*. 1990;8:715–724.
80. Campbell DJ, Kladis A, Duncan AM. Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension*. 1994;23:439–449.
81. Gomez RA, Lynch KR, Chevalier RL, Wilfong N, Everett A, Carey RM, Peach MJ. Renin and angiotensinogen gene expression in the maturing rat kidney. *Am J Physiol*. 1988;254:F582–F587.
82. Ingelfinger J, Zuo WM, Fon EA, Ellison KE, Dzau VJ. In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. *J Clin Invest*. 1990;85:417–423.
83. Yanagawa N, Capparelli AW, Jo OD, Fridel A, Barrett JD, Eggena P. Production of angiotensinogen and renin-like activity by rabbit proximal tubule cells in culture. *Kidney Int*. 1991;39:938–941.
84. Bruneval P, Hinglais N, Alhenc-Gelas F, Tricotet Y, Corvol P, Menard J, Camilleri JP, Bariety J. Angiotensin I converting enzyme in human intestine and kidney. Ultra-structural and immunohistochemical localization. *Histochemistry*. 1986;85:73–80.
85. Terada Y, Tomita K, Nonoguchi H, Marumo F. PCR localization of angiotensin II receptor and angiotensinogen mRNA in rat kidney. *Kidney Int*. 1983;43:1251–1259.
86. Ye M, Wysocki J, Naaz P, Salahat MR, LaPointe MS, Battle D. Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a nonreproductive combination? *Hypertension*. 2004;43:1120–1125.
87. Largo R, Gomez-Garre D, Soto K, Marron B, Blanco J, Gazpao RM, Plaza JJ, Egido J. Angiotensin-converting enzyme is upregulated in the proximal tubules of rats with intense proteinuria. *Hypertension*. 1999;33:732–739.
88. Li N, Zimpelman J, Cheng K, Wilkins JA, Burns KD. The role of angiotensin converting enzyme 2 in the generation of angiotensin 1–7 by rat proximal tubules. *Am J Physiol Renal Physiol*. 2005;288:F353–F362.
89. Hunt MK, Ramos SP, Geary KM, Norling LL, Peach MJ, Gomez RA, Carey RM. Colocalization and release of angiotensin and renin in renocortical cells. *Am J Physiol*. 1992;263:F363–F373.
90. Darby IA, Sernia C. In situ hybridization and immunohistochemistry of renal angiotensinogen in neonatal and adult rat kidney. *Crit Care Med*. 1995;23:197–206.
91. Robrwasser A, Morgan R, Dillon HF, Zhao L, Callaway CA, Hillas E, Zhang S, Cheng T, Inagami T, Ward K, Teveros DA, Lalouel JM. Elements of a paracrine tubular renin-angiotensin system along the entire nephron. *Hypertension*. 1999;34:1265–1274.
92. Sibong M, Gase J-M, Soudrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension*. 1993;21:827–835.
93. Nishiyama A, Seh DM, Navar LG. Renal interstitial fluid concentrations of angiotensins I and II in anesthetized rats. *Hypertension*. 2002;39:129–134.
94. Schiavone MT, Santos RAS, Brosnihan KB, Khosla MC, Ferrario CM. Release of vasopressin from the rat hypothalamic-neurohypophyseal system by angiotensin (1–7) heptapeptide. *Proc Natl Acad Sci U S A*. 1988;85:4095–4098.
95. Yamamoto K, Iyer SN, Chappell MC, Ganten D, Ferrario CM. Converting enzyme determines the plasma clearance of angiotensin (1–7). *Hypertension*. 1998;32:496–502.
96. Santos RA, Campagnolo-Santos MI, Andrade SP. Angiotensin (1–7): an update. *Regul Pept*. 2000;91:45–62.
97. Arima S. Role of angiotensin II and endogenous vasodilators in the control of glomerular hemodynamics. *Clin Exp Nephrol*. 2003;7:172–178.
98. Nakamura S, Averill DB, Chappell MC, Diz DI, Brosnihan KB, Ferrario CM. Angiotensin receptors contribute to blood pressure homeostasis in salt-depleted SHR. *Am J Physiol Regul Integr Comp Physiol*. 2003;84:R164–R173.



99. Burgelova M, Kramer HJ, Teplan V, Velickova G, Vitko S, Heller J, Maly J, Cervenkova L. Intrarenal infusion of angiotensin-(1-7) modulates renal functional responses to exogenous angiotensin II in the rat. *Kidney Blood Press Res*. 2002;25:20.
100. Ferrario CM, Averill DB, Brosnihan KB, Chappell MC, Iskandar SS, Dean RH, Dix DJ. Vasopressinase inhibition and Ang-(1-7) in the spontaneously hypertensive rat. *Kidney Int*. 2002;2:1349-1345.
101. Brosnihan KB, Neves LA, Joyner J, Averill DB, Chappell MC, Sarao R, Penninger J, Ferrario CM. Enhanced renal immunocytochemical expression of ANG-(1-7) and ACE2 during pregnancy. *Hypertension*. 2003;42:749-753.
102. Gilbert RE, Wu LL, Kelly DJ, Cox A, Wilkinson-Berka JL, Johnston CI, Cooper ME. Pathological expression of renin and angiotensin II in the renal tubule after subtotal nephrectomy: implications for the pathogenesis of tubulointerstitial fibrosis. *Am J Pathol*. 1999;155:429-440.
103. Zoja C, Donadelli R, Corna D, Testa D, Facchinetti D, Maffi R, Luzzana E, Colosio V, Bertani T, Remuzzi G. The renoprotective properties of angiotensin-converting enzyme inhibitors in a chronic model of membranous nephropathy are solely due to the inhibition of angiotensin II: evidence based on comparative studies with a receptor antagonist. *Am J Kidney Dis*. 1997;29:254-264.
104. Peters H, Border WA, Noble NA. Angiotensin II blockade and low-protein diet produce additive therapeutic effects in experimental glomerulonephritis. *Kidney Int*. 2000;57:1493-1501.
105. Hisada Y, Sugaya T, Yamanouchi M, Uchida H, Fujimura H, Sakurai H, Fukamizu A, Murakami IK. Angiotensin II plays a pathogenic role in immune-mediated renal injury in mice. *J Clin Invest*. 1999;103:627-635.
106. Lafayette RA. How does knocking out angiotensin II activity reduce renal injury in mice? *Am J Kidney Dis*. 2000;35:166-172.
107. Ma LJ, Nakamura S, Whitsitt JS, Marcantoni C, Davidson JM, Fogo AB. Regression of sclerosis in aging by an angiotensin inhibition-induced decrease in PAI-1. *Kidney Int*. 2000;58:2425-2436.
108. Pagtalunan ME, Olson JL, Meyer TW. Contribution of angiotensin II to late renal injury after acute ischemia. *J Am Soc Nephrol*. 2000;11:1278-1286.
109. Metzger R, Böhle RM, Pauls K, Eichner G, Alhenc-Gelas F, Danilov SM, Franke FE. Angiotensin-converting enzyme in non-neoplastic kidney diseases. *Kidney Int*. 1999;56:1442-1454.

## **EXHIBIT 4**

## First interim evaluation of piglet ARDS model

Sept. 15, 2006

Alexander Löckinger, Benedikt Tremel, Manfred Schuster, Hans Loibner

### Introduction

Recombinant human soluble ACE2 (rhACE2) produced under protein free conditions in CHO cells by Apeiron Biologics was administrated to piglets in an LPS-induced ARDS model at the premises of the University Hospital Innsbruck. ARDS was induced in 8 animals: 4 animals were treated with rhACE2 at a dosage of 100 µg/kg and compared to a control group also composed of 4 animals. All animals had exactly the same age, similar body weight and had the same genetic antecedents (Table 1).

ARDS was induced by continuous infusion of 50 µg/kg LPS for the whole duration of the experiment and further 1 - 3 LPS bolus injections of 50 µg/kg each. Average LPS quantity was 319 µg/kg and nearly equally distributed over both groups. RhACE2 was central venously injected at a dose of 100 µg/kg following the last LPS bolus injection and 120 minutes from the start of the continuous LPS infusion. Several hemodynamic parameters as well as pharmacokinetics were investigated.

	<b>Control group</b>				
Animal number	7	9	10	12	Average
LPS µg x kg <sup>-1</sup>	320	370	433	200	331±99
Animal weight kg	26	23	20	22	23±3
	<b>rhACE2 treated group</b>				
Animal number	11	13	14	15	
LPS µg x kg <sup>-1</sup>	392	280	247	308	307±22
Animal weight kg	24	18	21,5	19	21±3

Table 1: Animal disposition, group distribution and LPS dosage

### Tolerability and side effects

100 µg/kg rhACE2 administrated as bolus injection were well tolerated and did not show any apparent side effects like blood pressure drop or heart frequency increase.

### Pharmacokinetics

RhACE2 distribution and activity are still under investigation. First evaluation showed an ACE2 activity (using a substrate Mca-APK-(Dnp)-OH based fluorescence based activity assay) in serum and ascites which was not detected in baseline samples. No activity was detected in urine or lung lavage. Figure 1 displays the measured time dependent ACE2 activity in serum samples after an infusion of 100 µg/kg at time point 0. We used a one phase exponential decay fit and calculated a half life time of  $T_{1/2\alpha}=77$  minutes, corresponding to the half life time of the initial compound distribution phase (Figure 1). To properly determine the terminal serum half-life a considerably longer observation time will be needed.

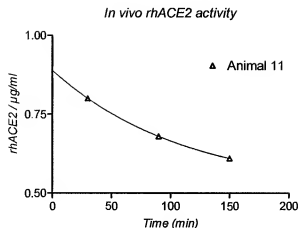


Figure 1: rhACE2 activity was measured in serum samples 30, 90 and 150 minutes after infusion of 100 µg/kg to an animal of 24 kg using fluorescent labelled tripeptide APK as substrate.

### Pulmonary Arterial Pressure (PAP)

PAP was measured online during the whole experiment and is summarized in Figure 2. From the time point of infusion of rhACE2 the control group showed a nearly 15% increase of PAP while the treated animals apparently stabilized on this baseline level or even showed a slight decrease. Difference between both groups was statistically significant at 60 ( $p < 0.06$ ), 120 minutes ( $p < 0.05$ ) and also at 150 minutes ( $p < 0.05$ ) after ACE2 infusion.

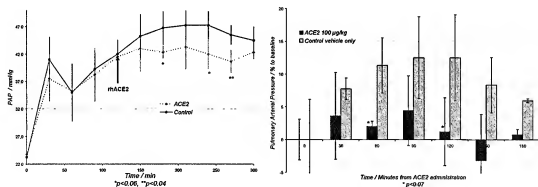


Figure 2: Pulmonary arterial pressure was monitored during the whole experiment. Average values of both groups are displayed (A). Variation from the time point of rhACE2 infusion (0 minutes) is displayed in percentages (B).

### Systolic Arterial Pressure (SAP)

SAP was also measured online and average values of both groups are displayed in Figure 3. The control group showed an increase up to 12% while after rhACE2 injection a stabilization and further 5% decrease was observed. The difference between both groups was statistically significant at 60 ( $p < 0.07$ ), 120 minutes ( $p < 0.03$ ) and also at 150 minutes ( $p < 0.07$ ).

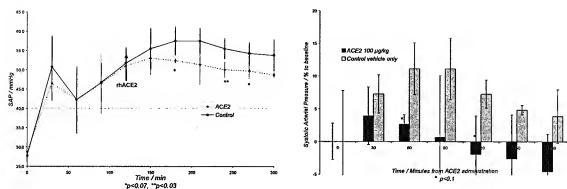


Figure 3: Systolic arterial pressure was monitored during the whole experiment. Average values of both groups are displayed (A). Variation from the time point of rhACE2 infusion (0 minutes) is displayed in percentages (B).

#### Arterial and venous $pO_2$ concentration

Oxygen concentration was measured in arterial and venous blood samples taken every 30 minutes. Values are displayed in Figure 4. Oxygen concentration decreased in arterial and venous blood in both groups. A potential stabilization of arterial as well as venous oxygen concentration in the group receiving rhACE2, which might be observed first in the venous, later in the arterial blood, is not statistical significant and will have to be confirmed in further experiments.

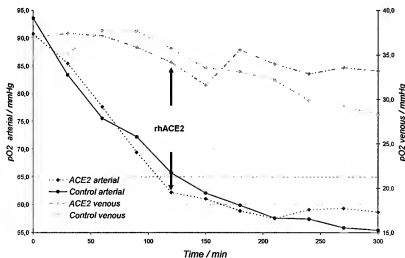


Figure 4: ARDS model: arterial and venous oxygen concentration measured in blood samples of animals treated with rhACE2 (blue curves) and control animals (black and grey curves).